Role of Protein Kinase C Zeta in Lipopolysaccharide-mediated Nuclear Factor Kappa B Aactivation and Transcriptional Aactivity in Kidney Epithelial Cells

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A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

2006

Program Authorized to Offer Degree:
Department of Public Health and Community Medicine
Environmental and Occupational Health Sciences

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Abstract

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NF-κB is a critical transacription factor that mediates cellular responses to invading pathogens. In the kidney, NF-κB's most important targets are the cytokines and chemokines, which regulate the scale of the inflammatory response by recruiting and facilitating immunocyte activity in the kidney, especially polymorphonuclear leukocytes. For induction of these genes, NF-κB must be translocated from the cytoplasm to the nuclear compartment, bind nuclear κB consensus elements and induce RNA polymerase loading at these sites.

In this work we show that in kidney proximal tubule cells, PKCζ acts upstream of multiple phases of NF-κB activation, and is a determinant of the profile of cytokines induced in response to LPS. We show that PKCζ interacts with the IKK complex directly and that inhibition of PKCζ's catalytic activity with either pharmacologic inhibitors or overexpression of a dominant negative PKCζ blocks IKK activity and subsequent NF-κB DNA binding. We also show that PKCζ regulates NF-κB transactivation not only through direct phosphorylation of serine 311 on p65, but also through a novel regulatory mechanism associated with p300 activation. The

culmination of PKCζ activity is a shift in the expression of NF-κB driven genes away from the proinflammatory genes, TNFα and Cox-2, to the inflammatory resolution genes IL-6 and A20.

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LIST OF ABBREVIATIONS

APKC	Atypical Protein Kinase C
AKT	PKB protein kinase B
CBP	Creb Binding Protein
COX-2	Cyclooxygenase 2
IκB	Inhibitor of Kappa B
IKK	Inhibititor of Kappa B Kinase
IL-1β	Interleukin 1β
IL-6	Interleukin 6
LPS	Lipopolysaccharide
MEKK	MEK Kinase
MSK1	Mitogen and Stress Activated Kinase 1
PDK1	3-Phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
RIP	Receptor Interacting Protein
TIR	Toll Like/ Interleukin 1 Receptor
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor α
TNF-R1	Tumor Necrosis Factor Receptor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRIF	Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β

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INTRODUCTION

Activation of the immune system is a critical step in the clearance of pathogens from the body, but aberrant activation of the immune system can result in a variety of diseases of the kidney, including sepsis, acute renal failure, interstitial fibrosis, lupus nephritis, or renal transplant rejection (reviewed in [1]). Initially discovered in B-cells, the pleiotrophic transcriptional activator, nuclear factor kappa B (NF-κB) stimulates the immune response through the induction of cytokines, chemokines and receptors associated with the inflammatory response in addition to prosurvival and proliferation genes and is known to be important to several pathological states (reviewed in [2]). According to the receptor profile expressed by individual cells, NF-κB can be activated by pathogen components such as double stranded RNA and lipopolysaccharide (LPS), or cytokines such as tumor necrosis factor, interleukins 1β and 6 or pharmacologic agents like phorbol [3-5]. We demonstrate for the first time that the atypical PKC, PKCζ, interacts with the NF-κB cascade at multiple points and that this interaction is correlated with a reduction in the inflammatory actions of LPS in rat kidney epithelial cells.

NF-κB activation involves two highly regulated steps, DNA binding and transactivation. The first step entails the translocation of NF-κB from the cytoplasm to the nucleus and the interaction of the leucine zipper domain of the p65 component with the DNA. The Inhibitor of κB (IκB) family proteins prevent the interaction of p65 with DNA in resting cells. Upon activation of the NF-κB pathways the IκB proteins are degraded subsequent to their phosphorylation by IKK and p65 is released in a conformation capable of binding DNA [5-7]. We found that PKCζ positively influences IKK activation and NF-κB interaction with DNA binding.

Upon binding the DNA, p65 must undergo several posttranslational modifications for NF-κB to efficiently induce transcription [8]. The transactivation process of NF-κB involves the phosphorylation of p65 on sites that recruit histone acetyl transferases (HAT) to the bound promoter [9-11]. The HATs acetylate p65 and the surrounding DNA, relieving local histone induced supercoiling and allowing for processive loading of RNA polymerase upstream of the transcription start site, a critical step in initiating mRNA production [12]. Herein we show that PKCζ enhances the ability of p65 to interact with p300, limits the activity of p300, reduces NF-κB transactivation and increases the expression of NF-κB mediated genes associated with inflammatory resolution.

These findings better describe the specific mechanism(s) utilized by rat kidney cells to regulate the two phases of NF-κB activation in response to LPS. Further research will be required to determine how these findings are applicable to specific human disease states and how PKCζ modulators might be used to intervene in human conditions.

CHAPTER 1: OBJECTIVES

RATIONALE

Nuclear factor kappa B (NF-κB) is a pleiotrophic transcriptional activator that is known to be involved in the inducible expression of a wide variety of genes, including those that mediate inflammation [13, 14]. While much attention has focused on the role of NF-κB in these functions in numerous cell types, relatively few studies have investigated signaling mechanisms underlying regulation of NF-κB activation by inflammatory agents in epithelial cells of renal tubular origin. Identification of such mechanisms is of increasing interest in light of emerging evidence that renal tubular epithelial cells play a central role in the etiology of both acute and chronic kidney diseases through expression of inflammatory mediators and attractants that are produced in association with NF-κB activation [15, 16].

NF-κB is readily activated in renal tubular epithelial cells by numerous pro-inflammatory agents including bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) [17, 18]. Recent investigations into the signaling mechanisms involved in NF-κB activation have identified members of the family of protein kinase C (PKC) as playing a key role in these processes [19]. Notably, the atypical isoform, PKCζ, has been shown to play a central role in NF-κB activation and/or transcriptional activity in various cell types [20]. Herein we show that kidney epithelial cells constitutively express PKCζ, and inhibition of PKCζ prevents NF-κB activation. Therefore, it is reasonable to hypothesize that PKCζ plays an important role in the regulation of NF-κB activation or NF-κB mediated gene expression in kidney cells.

In this work, we sought to define the role(s) of PKCζ in LPS mediated NF-κB DNA-binding and transcriptional activity in kidney epithelial cells. Additionally, we investigated the consequences of PKCζ activation or inhibition on the expression of specific NF-κB-regulated genes that are involved in kidney cell injury.

HYPOTHESES

a) PKCζ induces NF-κB DNA-binding through modulation of upstream activation kinetics, as in Figure 1.
b) PKCζ modulates NF-κB transactivation by phosphorylating p65 on serine 311 directly and by inducing IKK dependent phosphorylation of p65 on serine 536, as in Figure 2.
c) PKCζ mediated transactivation of NF-κB results in characteristic gene expression changes.

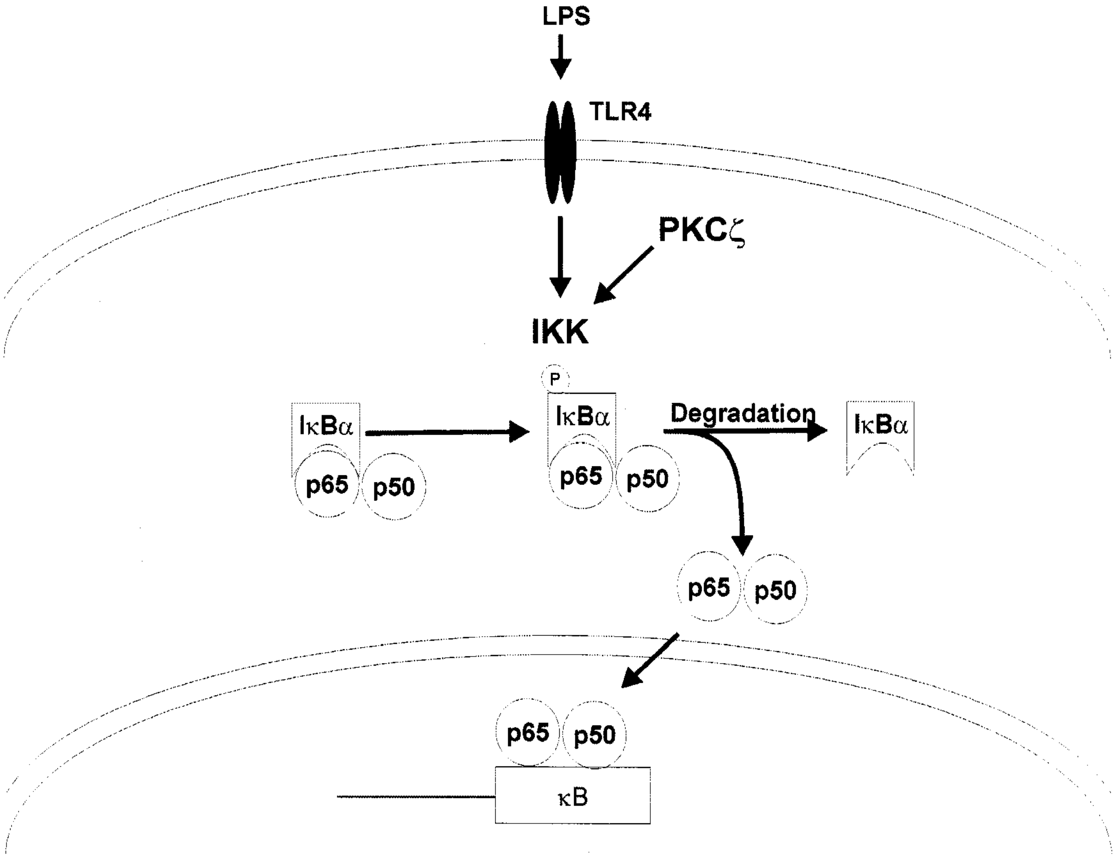

Figure 1. **PKCζ induces NF-κB translocation signaling through the upstream master kinase, IKK.** LPS activation of the TLR4 receptor at the plasma membrane of the cell results in activation of IKK. IKK phosphorylates IκBα, signaling it for degradation and releasing the p65/p50 dimer. p65/p50 then translocates to the nucleus where they bind to κB elements in the promoters of NF-κB target genes. Based on previous work, we hypothesize that PKCζ acts upstream of IKK as a necessary component for activation of the translocation cascade.

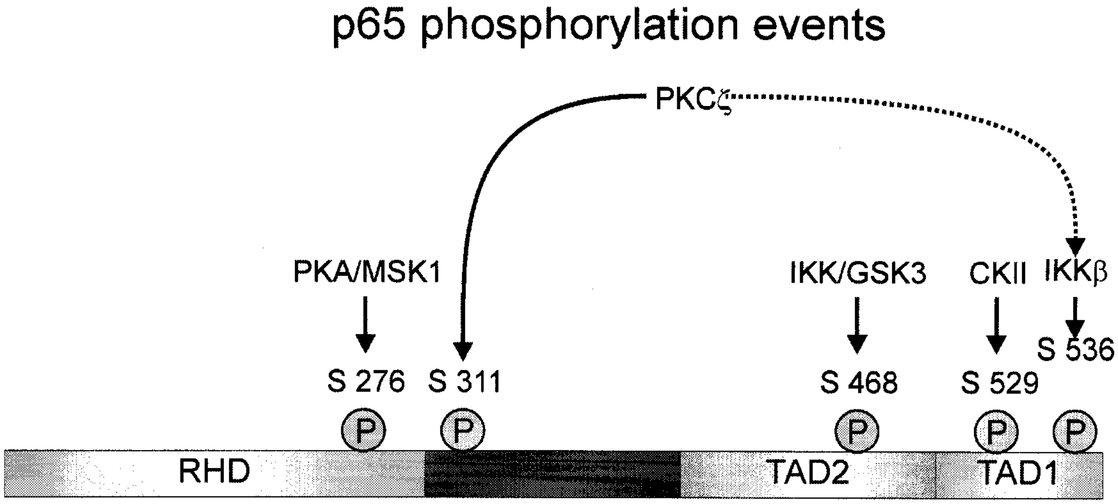

Figure 2. **p65 is phosphorylated at several sites after activation**. After release of IκBα several sites on p65 become phosphorylated. We show the implicated kinases and their site of action for each phosphorylation event known to regulate transactivation. PKCζ activates IKKβ in the translocation phase of activation, so we propose a similar function in transactivation, indicated by the dashed line [21].

SPECIFIC AIMS

I. Demonstrate that PKCζ induces NF-κB binding through activation of IKK.

A. Inhibit NF-κB DNA-binding with the specific cell permeable PKCζ pseudosubstrate.

B. Inhibit NF-κB DNA-binding with a plasmid based dominant negative inhibitor of PKCζ (PKCζDN).

C. Demonstrate that IκBα kinetics are disturbed in the presence of a PKCζ inhibitor.

D. Establish an IKK activity assay based on optimization of conditions described in other cell models.

E. Demonstrate that IKK activity is not increased in response to LPS when a PKCζ inhibitor is present.

F. Associate PKCζ activation and LPS induced NF-κB DNA-binding by demonstrating a temporal relationship where PKCζ activity precedes NF-κB activation.

II. Demonstrate that PKCζ is responsible for transactivation of NF-κB.

A. Demonstrate that PKCζ regulates NF-κB transactivation.

   1) Demonstrate that PKCζ activation is necessary for transactivation of NF-κB.

2) Establish a transactivation assay that directly measures the ability of the p65/p50 dimer to bind the histone acetyl transferase p300 by colocalization via immunoprecipitation.
3) Demonstrate that PKCζ regulates the colocalization of HAT activity with p65, consistent with the activation of NF-κB luciferase.

B. Demonstrate that PKCζ induces NF-κB transactivation through the phosphorylation of p65 on serine 311.
   1) Demonstrate that LPS induces p65 Ser 311 phosphorylation.
   2) Establish that PKCζ activity and p65 Ser 311 phosphorylation exhibit a temporal relationship.
   3) Demonstrate that blockage of PKCζ activity prevents LPS induced p65 Serine 311 phosphorylation.

C. Demonstrate that PKCζ induces NF-κB transactivation by increasing IKK mediated phosphorylation of p65 at serine 536.
   1) Demonstrate that LPS induces p65 Ser 536 phosphorylation.
   2) Establish that PKCζ activity and p65 Ser 536 phosphorylation exhibit a temporal relationship.
   3) Establish that IKK activity and p65 Ser 536 phosphorylation exhibit a temporal relationship.
   4) Demonstrate that prevention of PKCζ activity prevents LPS induced p65 Ser 536.

5) Demonstrate that prevention of IKK activity by the IKK inhibitor wedelolactone prevents LPS induced p65 Ser 536.
6) Establish that IKK activitation and NF-κB transactivation exhibit a temporal relationship.
7) Demonstrate that wedelolactone prevents NF-κB transactivation.

III. Demonstrate that modulation of PKCζ results in changes in gene expression downstream of NF-κB. Identify NF-κB genes induced by LPS treatment of NRKs.

B. Demonstrate the PKCζ inhibition prevents expression of these genes.

C. Demonstrate that PKCζ overexpression increases expression of these genes.

CHAPTER 2: BACKGROUND

KIDNEY DISEASE AND LIPOPOLYSACCHARIDE

Regulation of the inflammatory response is a critical determinant in the outcome of multiple kidney diseases. The kidney seems particularily sensitive to inflammatory damage, as survival is often correlated with kidney function in cases of bacterial septicemia [22, 23]. Lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria and some algae, activates innate immunity through toll like receptor 4 (TLR-4) [24-26]. Activation of this receptor is associated with normal immune response after pathogen infection, but high amplitude or long-term activation of TLR-4 has been implicated in several human diseases [27]. Within the kidney, aberrant activation of TLR-4 has been associated with sepsis leading to acute renal failure and renal fibrosis, whereas deficiency in Myd88, a critical intermediate of TLR signaling, reduces renal transplant rejection [1, 28]. The critical downstream effector of TLR4 signaling is the transcription factor NF-κB [29]. NF-κB signaling helps to maintain kidney function by inducing survival genes in the damaged kidney but also can accelerate kidney failure by inducing the release of cytokines responsible for inducing inflammatory changes in the kidney and can also exacerbate ischemia/reperfusion injury [30-32]. While it is known that cytokine induced immune cell infiltration of the kidney is correlated with poor outcome in many renal diseases, the specific mechanisms governing the activation of NF-κB in renal cells is poorly defined [33]. Furthermore, recent studies have demonstrated that environmental agents such as mercury can exacerbate the death of renal proximal tubule cells and thus increase the severity of injury in response to mercury, while inhibiting the crucial inflammatory mediator NF-κB [17, 18]. Insufficient knowledge of the kinetics of inflammation related changes in these cells underscores the need for additional research into the mechanisms controlling this outcome. In this work, we characterize the upstream kinetics of NF-κB in response to LPS and show that NF-κB gene induction is likely to be an important determinant of inflammation in a rat kidney epithelial cell line.

NUCLEAR FACTOR KAPPA B ACTIVATION

Nuclear factor kappa B (NF-κB) is a plieotrophic transcriptional activator that is known to be involved in the expression of multiple genes involved in the inflammatory response [13, 34]. In most mammalian cells, NF-κB is a heterodimer comprised of a 50 kilodalton protein (p50) and a 65 kilodalton protein referred to as RelA or p65 [35]. Under nonactivating conditions, NF-κB is prevented from accumulating in the nucleus and binding DNA by the inhibitor of κB (IκBα). Upon stimulation by many factors, inhibitor of IκB kinase (IKK) is activated and it subsequently phosphorylates IκBα, thus targeting it for degradation by the proteosome. IκBα degradation allows NF-κB translocation to the nucleus where it binds κB gene promoter elements, as depicted in Figure 1 [13, 36]. The recent finding that IRAK-1, an upstream regulator of NF-κB (Figure 3) was a partial predictor of survival in cases of sepsis highlights the importance of NF-κB in this model [37].

SIGNAL TRANSDUCTION UPSTREAM OF IKK

Potent activation of NF-κB can be accomplished by lipopolysaccharide (LPS), interleukin 1β (IL-1β), or tumor necrosis factorα (TNFα) in rat kidney epithelial cells [17, 18]. LPS and IL-1β utilize different members of the same TLR/interleukin 1 receptor (TIR) superfamily of receptors, and subsequently display very similar downstream kinetics, as displayed in Figure 3 [38, 39]. Upon activation of the toll like receptor-4 (TLR4) or interleukin 1 receptor (IL-1R) by their respective ligand (LPS & IL-1β), both receptors recruit the shared adaptor, Myd88 and its scaffold proteins IRAK-1 and IRAK-4 to the active receptor complex [40-42]. IRAK-1 becomes hyperphosphorylated as a result of IRAK-4 activity, and facilitates the binding of TRAF-6 to the complex [43, 44]. TRAF-6 contains a Ring domain that displays E3 ubiquitin ligase activity, which once associated with the complex rapidly attaches ubiquitin in K63 linked strands to itself and IRAK-1 [45, 46]. K63 linked ubiquitin does not signal degradation, but instead recruits the TAB1/TAB2/TAK1 and IKKβ/IKKα/IKKγ complexes through their ubiquitin binding domains [47-49]. Once the complex is assembled, MEKK3 activates IKKβ, the kinase responsible for IκBα phosphorylation, degradation and subsequent activation of the canonical NF-κB cascade (Figure 1)[50, 51]. This cascade is inhibitied at multiple levels by the action of the A20/ABIN complex. The A20/ABIN complex inhibits the activation of NF-κB by preventing TRAF6 ubiquination of the signalasome, and facilitating the degradation of IRAK1 by adding ubiquitin in the K47 linkage [52, 53]. A20 induced rearrangement and IRAK1 degradation are required for the termination of LPS induced NF-κB activity [52].

The overall pathway is very similar downstream of TNFα induced activation of TNFα receptor-1 (TNFR-1) (Figure 4) (reviewed in [54, 55]. TNFR-1 recruits TRAF2 through the adaptor TRADD and scaffold receptor interacting protein-1 (RIP-1). RIP-1 allows for multimerization of TRAF2, a homologue of TRAF6, facilitating its ubiquitin ligase activity and recruitment of TAK1 and IKK [56, 57]. Strangely, LPS can also recruit RIP-1 through the adapator TRIF, when its high affinity adaptor, Myd88, is knocked out [58-60]. The same mechanism of termination functions in the TNF pathway as the LPS pathway, except that A20 targets the ubiquitin chains on RIP-1 for rearrangement and degradation in lieu of IRAK-1 [61].

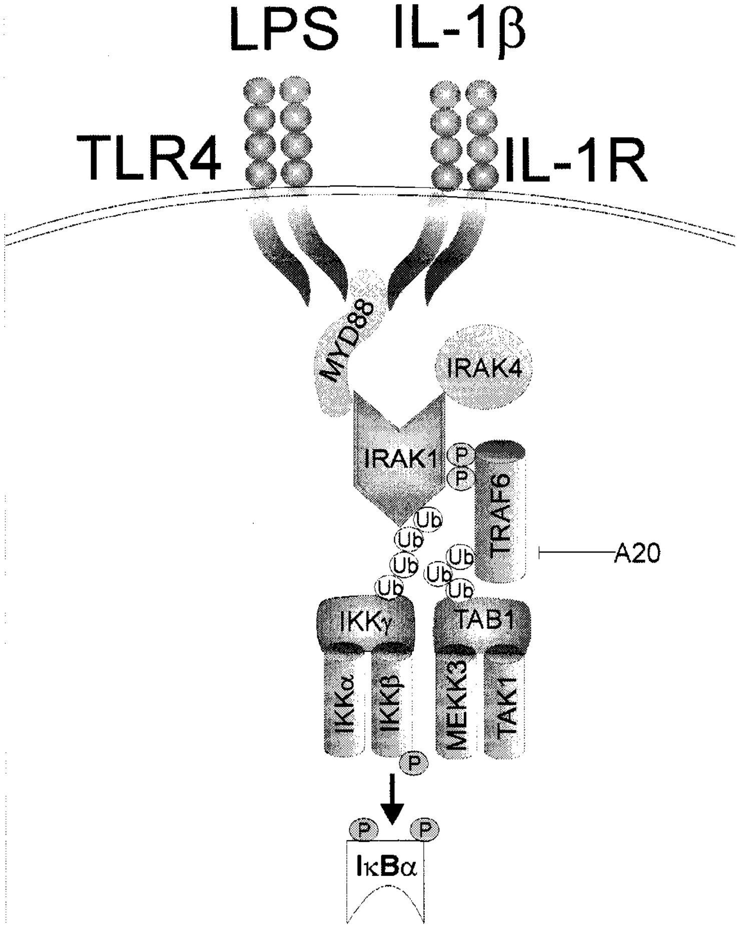

Figure 3. **TIR signaling to IKK.** LPS and IL-1β stimulate signaling through the TIRs, TLR4 or IL-1R. The adaptor proteins Myd88 and IRAK1 recruit and activate the ubiquitin ligase domain of TRAF6, resulting in ubiquination of IRAK1. The ubiquitin chains interact with IKKγ and TAB through their ubiquitin binding domains which brings the activator kinases MEKK3 and TAK1 into close proximity with the effector kinases IKKα and IKKβ. Upon activation, IKKβ phosphorylates IκBα leading to p65 translocation from the cytoplasm to the nucleus. A20 terminates IKK activity by rearranging IRAK1 ubiquitin, thus targeting it for degradation.

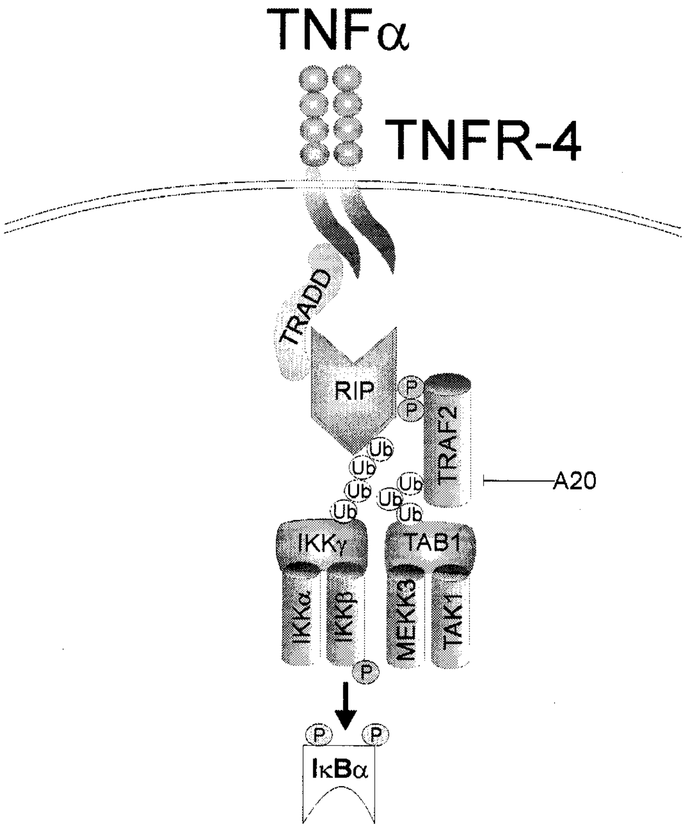

Figure 4. **TNF signaling to IKK.** Stimulation of the TNF receptor causes activation of the cascade responsible for IKK activation. TRADD and RIP act as scaffolding for the recruitment and activation of TRAF2. TRAF2 mediated ubiquitination recruits IKKγ and TAK1 into a complex where MEKK3 and IKKβ are in close proximity. MEKK3 phosphorylates IKKβ activating it, resulting in phosphorylation of IκBα.

NUCLEAR FACTOR KAPPA B TRANSACTIVATION

Nuclear NF-κB is capable of binding DNA through its leucine zipper domain, but gene induction requires the recruitment of additional factors to p65 in a process known as transactivation [61-64]. Transcription requires that RNA polymerase both load onto and progressively scan the promoter region of a gene until it finds a transcription start site, where upon RNA polymerase recruits the remaining transcriptional machinery and initiates mRNA production [65]. Targeted acetylation of histones results in local relief of DNA supercoiling and loading of RNA polymerase (Figure 5) [12]. NF-κB, through the p65 subunit, recruits the acetyl transferases, p300 or CBP, in the final step of transactivation before transcription initiation [36, 66].

The modification of p65 such that it can associate with acetyl transferases is necessary for LPS mediated induction of several NF-κB target genes [15]. Post-translational modification of p65 is necessary for efficient CBP or p300 recruitment to the promoter of TNFα or IL-6 in response to LPS [67]. Phosphorylation of p65 at serine 276 by either protein kinase A (PKA) or mitogen and stress activate kinase 1 (MSK-1) is compulsory for recruitment of the CBP (Figure 6) [66, 68]. Likewise, phosphorylation of serine 536 by IKKβ in the transactivation domain is required for full activation of several NF-κB mediated genes, although recent evidence suggests that this site may regulate p65's nuclear kinetics [36, 69-71]. Phosphorylation of p65 on Serines 311 and 529 by protein kinase C ζ and casein kinase II, respectively, is not required for transactivation, but blocking phosphorylation at these sites severely impairs NF-κB driven gene expression [64, 72]. Dephosphorylation of serine 486, a target of glycogen synthase kinase 3β (GSK3β) in resting cells and of IKKε in active cells has been proposed as both a negative and positive regulator of transactivation, an outcome that is apparently system specific [73-75]. A thorough review of these sites is provided in Schmitz et al. [20].

NF-κB transactivation is also regulated by factors independent of the phosphorylation of the p65 subunit. Acetylation and ubiquitination of the p65 subunit regulates the duration of nuclear NF-κB activation and the induction of genes after stimulation [11, 76-78]. Furthermore, protein disulfide isomerase suppresses the transactivation of NF-κB without affecting either DNA binding or phosphorylation of p65 [79]. Mechanisms independent of p65 have also been identified to regulate NF-κB transactivation. For example, in IKKα–/– MEFs, p65 binds the DNA normally, but an entire class of genes is not induced in these cells [80, 81]. IKKα is important in the p65 dependent expression of IL-6, as it translocates to the nucleus and phosphorylates histone H3 directly, resulting in uncoiling of DNA in the region close to the IL-6 promoter [82]. Furthermore, inhibition of AKT, JNK and p38 decrease NF-kB transactivation, independent of p65 phosphorylation [83, 84].

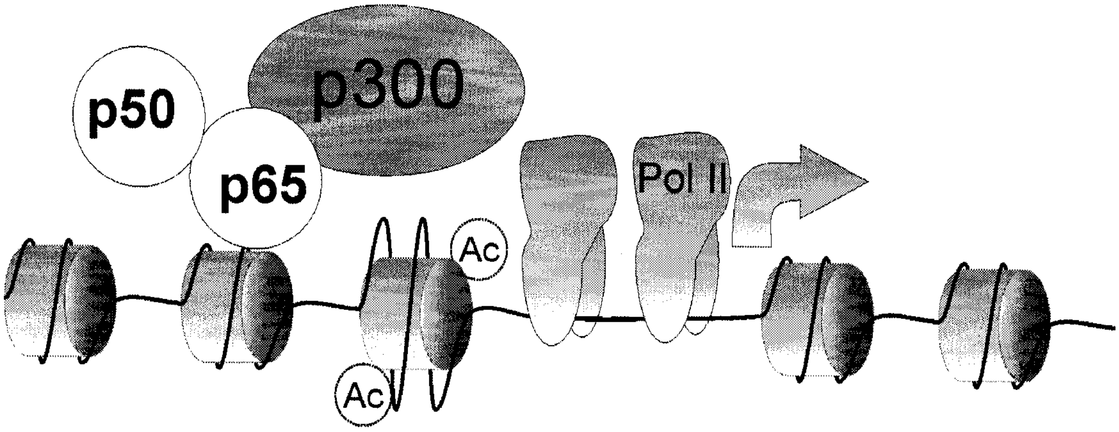

Figure 5. **p300 must be recruited to sites of active transcription.** After binding DNA, p65 must recruit p300 to the transcription starts site to induce gene expression. p300 acetylates histones resulting in the relaxation of the local DNA and exposure of RNA polymerase binding sites. Upon binding, RNA polymerase transcribes nascent mRNA [12].

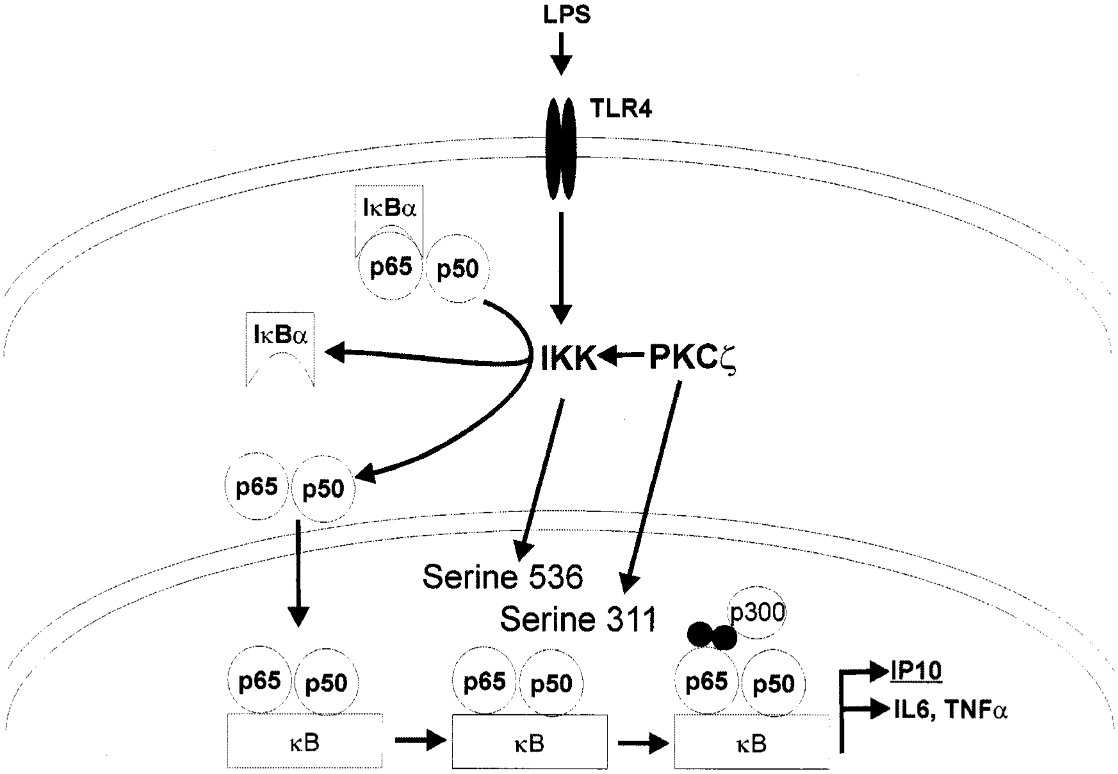

Figure 6. **p65 is directly phosphorylated by PKCζ, at serine 311, but not at serine 536.** After binding DNA, p65 must be phosphorylated at multiple sites to facilitate the interaction with the histone deactylase, p300. After interacting with p300 the local DNA is relaxed and transcription of target genes, both early and late (underlined) can be activated. Previous studies have demonstrated that PKCζ is capable of phosphorylating p65 directly at serine 311, but this has not been observed for serine 536. Instead, IKK phosphorylates p65 at serine 536, which increases NF-κB translocation and transactivation.

THE PROTEIN KINASE C FAMILY

The protein kinase C (PKC) superfamily is composed of serine threonine kinases in three subgroups defined by their mechanism of activation. The classical PKCs (PKCα, β_I, β_{II} and γ) require an increase in both intracellular calcium and diacylglycerol to induce catalytic activity, whereas the novel isoforms (PKCδ, ε, θ and η) only required diacylglycerol and not calcium. The atypical isoforms (PKCζ and ι/λ) require neither calcium nor diacylglycerol, but instead are activated by acidic lipids, including phosphatidic acid, ceramide, arachidonic acid and sphingosine-1-phosphate (for family review see [85-87], figure 7). Upon binding to their respective lipid, the PKCs translocate to the plasma membrane, are phosphorylated by PDK1 in their regulatory domain, and subsequently undergo autophosphorylation. The recruitment of PKCζ to the plasma membrane by the generation of ceramide after stimulation with LPS is an integral step in activation, as maturation of the PKCs is facilitated by the membrane bound kinase, PDK1 [88-91]. PDK1 phosphorylates PKCζ in the autoregulation loop, releasing this domain from the binding pocket and allowing for efficient interaction of the substrate with the catalytic center [92-94]. PKCζ displays very high activity upon activation, but also has a shortened half life in the cell due to destabilization of the protein after activation [95].

PROTEIN KINASE C ζ

PKCζ is an atypical PKC that represents a potential point of crosstalk between pathways as diverse as cytoskeletal regulation, insulin signaling, tight junction maintenance, survival, proliferation and inflammation [19, 96-103]. PKCζ is of particular interest in LPS induced sepsis because it prevents the loss of the epithelial tight junctions during inflammation by limiting NF-κB action [104]. The loss of the epithelial tight junction is necessary for LPS induced shedding of epithelial cells into the lumen, a primary cause of kidney failure and death during sepsis [104-106]. This finding is even more compelling considering that PKCζ is required for NF-κB activation. Inhibition of PKCζ activity by dominant negative PKCζ or knockout prevents the nuclear accumulation of p65 by blocking the activation of IKK in several models indicating a necessary role for PKCζ in the signalasome [107-109]. Furthermore, PKCζ is required for the phosphorylation of p65 on serine 311, which when blocked greatly decreases NF-κB gene induction [72, 110, 111]. Thus PKCζ has both positive and negative roles in NF-κB signaling [15, 104, 112]. While the inflammatory role of PKCζ has received considerable attention in other cell types, the mechanisms through which PKCζ regulate NF-κB in renal proximal tubule cells are undescribed.

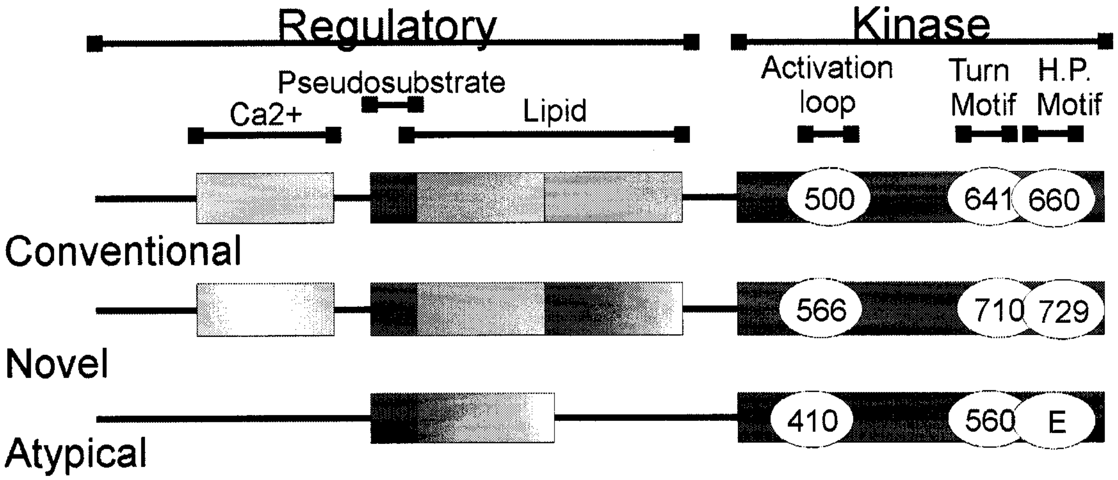

Figure 7. **Structure of the members of the PKC family.** The PKC family members share several similarities in structure. The regulatory domains of the convential and novel PKCs are almost identical, except that the novel PKCs lack a calcium binding pocket and thus do not react to calcium and are exclusively controlled by the binding of diacylglycerol to the lipid binding domain. The atypical PKCs, like PKCζ, have no calcium binding domain and a lipid binding domain that binds acidic lipids instead of diacylglycerol. Each kinase has a pseudosubstrate domain that blocks the activity of the kinase domain until the kinase is matured by phosphorylation in the activation loop. PDK1 phosphorylation of this domain releases the pseudosubstrate domain, allowing for full activity of the kinase.

CHAPTER 3: MATERIALS AND METHODS

MATERIALS

Reagents. All drugs and chemicals were obtained from standard commercial sources and met cell culture and molecular biology requirements. The myrisolated PKC pseudosubstrates and *Escherichia coli* serotype 026:B6 derived lipopolysaccharide were from Sigma (Sigma-Aldrich Co., St. Louis, MO).

METHODS

Cell culture and Treatment. The NRK52E cell line (ATCC CRL 1571) was acquired from American Type Culture Collection (Rockville, MD) and was propogated in Dulbecco's modified eagle medium (DMEM) with 4.5g/L glucose, pyruvate and glutamine supplemented with 5% heat inactivated new born calf serum, 2% fetal calf serum (Invitrogen, Carlsbad, CA) plus 100μ/ml penicillin and 100μg/mL streptomycin (Sigma). Cells were allowed to come to full confluence before being passed into a new 75cm^2 vent-cap tissue culture flasks (Nunc, Rodchester, NY). For treatment, the cells were passed into either 100mm^2 dishes, 60mm^2 dishes, 6well plates or 24well plates 2-3days prior to treatment and were treated when they covered 95% of the surface area. Treatments were administered to a final concentration of no greater than 1% of the total volume via direct addition of the dissolved substrate to the medium and careful mixing. Cells were harvested at times indicated after LPS treatment.

Nuclear Extraction. Nuclear lysates were obtained by the previously described protocol [113] . After treatment, cells were washed twice in ice cold PBS, and then scraped into 250μL of PBS containing Protease Inhibitor Cocktail (Sigma). The cells were spun at 15000xG for 15s at 4°C and the supernatant was removed. The cytosolic proteins were removed by resuspending the pellet in 30μL of Buffer A containing 0.1% NP-40 with a 10m incubation on wet ice. The nuclei were then pelleted by centrifugation at 15000xG for 10m at 4°C. The resulting supernatant comprised the cytosolic protein mass and was saved for analysis when appropriate. In order to achieve efficient lysis of the nuclear components, the cells were incubated for 10m at 4°C in Buffer C. Cellular debris was cleared by centrifugation at 15000xG for 10m at 4°C. The protein concentration of the resulting supernatant was quantified using the Bradford protein assay (Biorad, Hercules, CA).

Electromobility Shift. NF-κB EMSA was carried out as in Dieguiez-Acuna et al. [113]. Briefly, 5μg of nuclear extract was added to the EMSA reaction buffer containing T4 kinase mediated [γ-P^{32}]-labeled κB oligo (Promega, Madison, WI) to load 50,000/counts per well. The samples were gently mixed, centrifuged and then the binding reaction was carried out at 37°C for 30m. Electrophoresis was carried out on a nondenaturing TBE gel (Invitrogen). For quantitation the gels were dried for 45m at 80°C, and exposed to Biomax film (Kodak) overnight at -80°C.

Transfection. Cells were transfected using the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturers protocol. In brief, cells were seeded to achieve 50-80% confluency on the day of transfection. DNA was diluted in TE buffer. Enhancer solution was added and the reaction was allowed to stand at 25°C for 2m.

Effectene reagent was added and lipid vesicles were allowed to form for 10m. The plasmid solution was then added to the cells overnight. Efficiency of transfection was assessed by checking the number of cells with vacuoles. For stable transfection, mixed colonies were selected with geneticin (Invitrogen) across 3 weeks before culturing for experiments. The plasmid expressing IKKβ-DN was a gift of Dr. Cecilia Giachelli, University of Washington, Seattle. Plasmids containing a construct composed of a 4X tandem repeat of the NF-κB promoter response element inserted upstream of the coding region of a firefly luciferase gene in pGL2-basic [p4x-NF-κB-luc], human p65 cloned into pCDNA3, as well as the control vector [pCDNA 3] were provided by Dr. Nelson Fausto, University of Washington, Seattle. The HA tagged wildtype PKCζ and dominant negative PKCζ expression vectors were provided by Dr. Jorge Moscat, Universidad Autónoma de Madrid, Madrid. The pFC-MEKK1 was purchased as part of the Pathdetect NF-κB cis-reporting system (Stratagene, La Jolla, CA). The p65/galactosidase fusion protein reporter system, which is comprised of a p65/gal-4 fusion protein expression vector and a luciferase construct with a binding sequence for galactosidase, was a gift of Dr. Zhengui Xia, University of Washington, Seattle. The p300/galactosidase reporter system was comprised of the same luciferase system as above, and a p300/gal-4 fusion protein expression vector provided by Dr. Tony Krumm, University of Washington, Seattle.

Site directed mutagenesis. Site directed mutagenesis was carried out on the p65 pCDNA3 construct with the QuikChange® Site-Directed Mutagenesis Kit, according to the manufacturers protocol (Stratagene). The S311A conversion was carried out with the forward primer 5'-GACATATGAGACCTTCAAGGCCATCATGAAGA-AGAGTCC-3' and the reverse primer 5'-GGACTCTTCTTCATGATGGCCTTGA-AGGTCTCATATGTC-3'. The S536A conversion was carried out with the forward primer 5'-GATG-AAGACTTCTCCGCCATTGCGGACATGG-3' and the reverse primer 5'-CCATGTC-CGCAATGGCGGAGAAGTCTTCATC-3'. We confirmed conversion by sequencing of three independently selected clones.

Plasmid Maintenance. All plasmids were generated by bacterial amplification followed by the Endofree Maxi Plasmid Purification Kit (Qiagen). DH5α (Qiagen) were transfected by heat shock and allowed to recover for 1h before being plated on antibiotic containing agar plates. Single colonies were chosen and grown up under antibiotic selection at 37°C for 12h, after which, the cultures were centrifuged and the plasmid purified from the bacterial lysates.

Luciferase Assay. For transactivation assays cells were transfected with 400ng of κB luciferase and 20ng of CMV Renilla Luciferase per well and were harvested in Passive Lysis Buffer (Promega) at 4h post-treatment unless otherwise stated. For gal-4 fusion reporter systems, 100ng of the gal-4 luciferase was contransfected with 25ng β-galactosidase fusion construct per well and harvested in Passive Lysis Buffer 4h post LPS stimulation. Protein concentrations were measured by the Biorad protein assay. Equal protein and equal volume samples were analyzed using the Dual Luc kit (Promega) as recommended. Galactosidase activity was determined according with the β-galactosidase enzyme assay system (Promega). Luminescence measurements were taken on a PlateLumino (Phenix, Hayward, CA) and colorimetric measurements were taken on a Spectramax190 (Molecular Devices, Union City, CA).

Cellular Fractionation. For analysis of membrane bound fractions, cells were harvested by scraping into ice cold PBS and then pelleted at 1000g. After decanting the PBS, the pellets were resuspended in homogenization buffer (HB) (250mM sucrose, 10mM Tris, 1mM EDTA, 200nM DTT, pH 7.4 containing protease and phosphatase inhibitors) and manually homogenized by passing cell pellet 5times through a 25 5/8 gauge needle. The nuclei were pelleted by centrifugation for 10min at 1000g & 4C. Subsequent resuspensions in HB followed, with centrifugations at 10,000g, 15,000g and 100,000g to isolate the mitochondrial, lysosomal and microsomal fractions, respectively. The membrane bound proteins were resuspended in HB supplemented with 0.5% SDS and cleared by centrifugation at 10000g. Analyses were performed by Western blot.

Western blot analyses. Cells were lysed in Solution I of the DiDonato Assay, as follows: 40mM β-glycerophosphate, 250mM NaCl, 20mM NaF, 1mM Na3VO4, 20mM p-nitrophenylphosphate (PNPP), 1mM dithiothreitol (DTT), 0.1% Nonidet P-40, 0.1% Triton-X100, 1mM phenylmethysulfonyl fluoride, 1µl/ml Protease inhibitor cocktail, and 20mM HEPES-NaOH, pH 7.6 supplemented with 1µL/mL of phosphatase inhibitor cocktails 1 & 2 (Sigma). Total protein concentration was determined by the Bradford protein assay (Biorad). The Western blotting protocol was a modification of previously described method [113]. Briefly, 10-40 μg of protein were separated by electrophoresis, transferred onto polyvinylidine diflouride membranes, and then probed with the appropriate primary antibody. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) as described by the manufacturer. The phospho32/36 anti IκBα & IκBα antibodies were obtained from Cell Signaling Technology (Beverly, MA). All secondary antibodies and the Cox-2, iNOS, and p65 primary antibodies were acquired from Santa Cruz (Santa Cruz, CA). Anti-A20 antibody was obtained from Active Motif (Carlsbad, CA). Blots were stripped with ChemiStrip (Chemicon International, Inc., Temecula, CA) and reblotted.

IKKβ Activity Assay. IKKβ activity was determined by a modification of the assay described by DiDonato [114] as follows. Cells were lysed in modified Solution I as described above. The lysates were incubated on ice for 10 minutes, sonicated and cleared by centrifugation at 15,000G for 10 minutes. IKKβ was immunoprecipitated from 500μg total protein by tumbling for 2h at 4°C with either 1μg anti-IKKβ (Millipore, Billerica, MA) or 1.5μg anti-IKKα (Santa Cruz) per sample. The tubes were centrifuged for 30 sec at 15,000G and washed 4 times, twice in Solution I and twice in kinase buffer (20 mM β-glycerophosphate, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM PNPP, 50 mM NaCl, 2 mM DTT, 1 μl/ml protease inhibitor cocktail and 20 mM HEPES-NaOH, pH 7.6). After each wash, the beads were pelleted, and the supernatant was completely removed. The resultant pellets were incubated in a shaking water bath for 10 min at 37˚C in 25 μl kinase buffer supplemented to 0.1 μg/mL of GST-IkBα (Santa Cruz), 20 μM ATP and 5 μCi of [γ-^{32}P]ATP. Samples were loaded with LDS sample buffer (Invitrogen) and resolved by gel electrophoresis. Equal loading of IKK was confirmed by Coomassie Blue staining (Sigma).

mRNA collection and analysis. mRNA was harvested from 100mm^2 dishes using 1mL Trizol® (Invitrogen), according to the manufacturers instruction. The resulting concentration was determined by the ratio of absorbance of the sample at 260nm/280nm. Any experiment with greater than 10% of samples exhibiting a 260nm/280nm ratio of ≤1.7 or ≥1.9 were discarded. DNA was removed from the samples by adding 2μg of mRNA to a final volume of 1.8U/μL RNAsin (Promega), 9.1mM DTT (Invitrogen) and 0.05U/μL DNAse I (Roche, Indianapolis, IN) for 30m at 37°C followed by 5m at 70°C. Subsequently cDNA was synthesized by first hybridizing 1mg/mL random nine-mers with the RNA for 5m at 70°C followed by 10m at 0°C and then running the samples at 45°C for 120m and 70°C for 5m with final concentrations of 1xfirst strand buffer (Invitrogen), 7.5mM DTT, 0.5mM dNTPs (Roche), 5U superscript II (Invitrogen).

The Functional Genomics Laboratory at the University of Washington developed fluorogenic 5' nuclease-based assays to quantitate the mRNA levels of specific genes. For gene expression measurements of IL-6, TNF alpha and β-actin, 4μL of cDNA were included in a PCR reaction (16μL final volume) that also consisted of the appropriate forward (FP) and reverse (RP) primers at 438nM each, 188nM TaqMan probe and 1X TaqMan® Fast Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems Inc., Foster City, CA). The PCR primers and the dual-labeled probes (6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA)) for all genes were designed using ABI Primer Express v.1.5 software (Applied Biosystems Inc., Foster City, CA). All oligo sequences are listed in Table 1. Amplification and detection of PCR amplicons were performed with the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) with the following PCR reaction profile: 1 cycle of 95°C for 15 sec, 40 cycles of 95°C for 1 sec, and 60°C for 20 sec. β-actin amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and β-actin gene expression levels were utilized as an internal control to normalize the data.

TABLE 1. Sequences for PCR primers and probes.

Gene	Sequences
Beta-actin	FP: 5′- CCCTAAggCCAACCgTgAAA -3′ RP: 5′- ACgACCAgAggCATACAgggA -3′ Probe: 5′-6FAM-ATgACCCAgATCATgTTTgAgACCTTCAACAC -TAMRA-3′
Rat IL-6	FP: 5'-CCAACTTCCAATgCTCTCCTAATg-3' RP: 5'-gAAATTCTTCAAgTgCTTTCAAgATgAg -3′ Probe: 5'-6FAM-ATGGTCTTGGTCCTTAGCCACTCCTTCTGTG TAMRA-3'
Rat TNFa	FP: 5'-AgACCCTCACACTCAgATCATCTTCT-3′ RP: 5'-CTCCgCTTggTggTTTgCTA-3' Probe: 5'-6FAM-ACTCgAgTgACAAgCCCgTAgCCCAC-

	TAMRA -3'

Statistical Analyses. Results represent the mean ± SD of 3 or more separate experiments that were conducted in duplicate. Analysis of differences between treatment groups was determined using a two-tailed *t*-test. The level of significance was chosen at $p < 0.05$. Analysis of differences when 3 or greater groups were analyzed was determined by a two-tailed ANOVA with bonferroni correction. Post hoc analysis with Dunnett's t-test was used when comparing multiple treatments to a single control.

CHAPTER 4: RESULTS

PKCζ INHIBITION ATTENUATES NF-κB ACTIVATION

In previous studies using the normal rat kidney epithelial cell line (NRK), our laboratory has demonstrated that NF-κB is readily activated in response to numerous pro-inflammatory agents including bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) [17, 18]. From a screen against potential modifiers of cytokine mediated NF-κB, we specifically identified atypical protein kinase C, PKCζ, signaling as a necessary component of the NF-κB cascade in these cells. As shown in Figure 8, we were able to block lipopolysaccharide (LPS) induced NF-κB-DNA binding with 50μM of a myrisolated, cell permeant PKCζ pseudosubstrate inhibitor (PKCζi) that mimics the autophosphorylation domain of PKCζ, but not with pseudosubstrate inhibitors to the classical and novel isoforms of PKC. Transient transfection with 1μg/100mm^2 of a kinase dead version of PKCζ (PKCζDN) previously reported to act as a dominant negative, resulted in full inhibition of LPS (1μg/mL) induced NF-κB-DNA binding up to 1h post treatment (Fig 9). These experiments demonstrate that inhibitors of PKCζ block NF-κB-DNA binding.

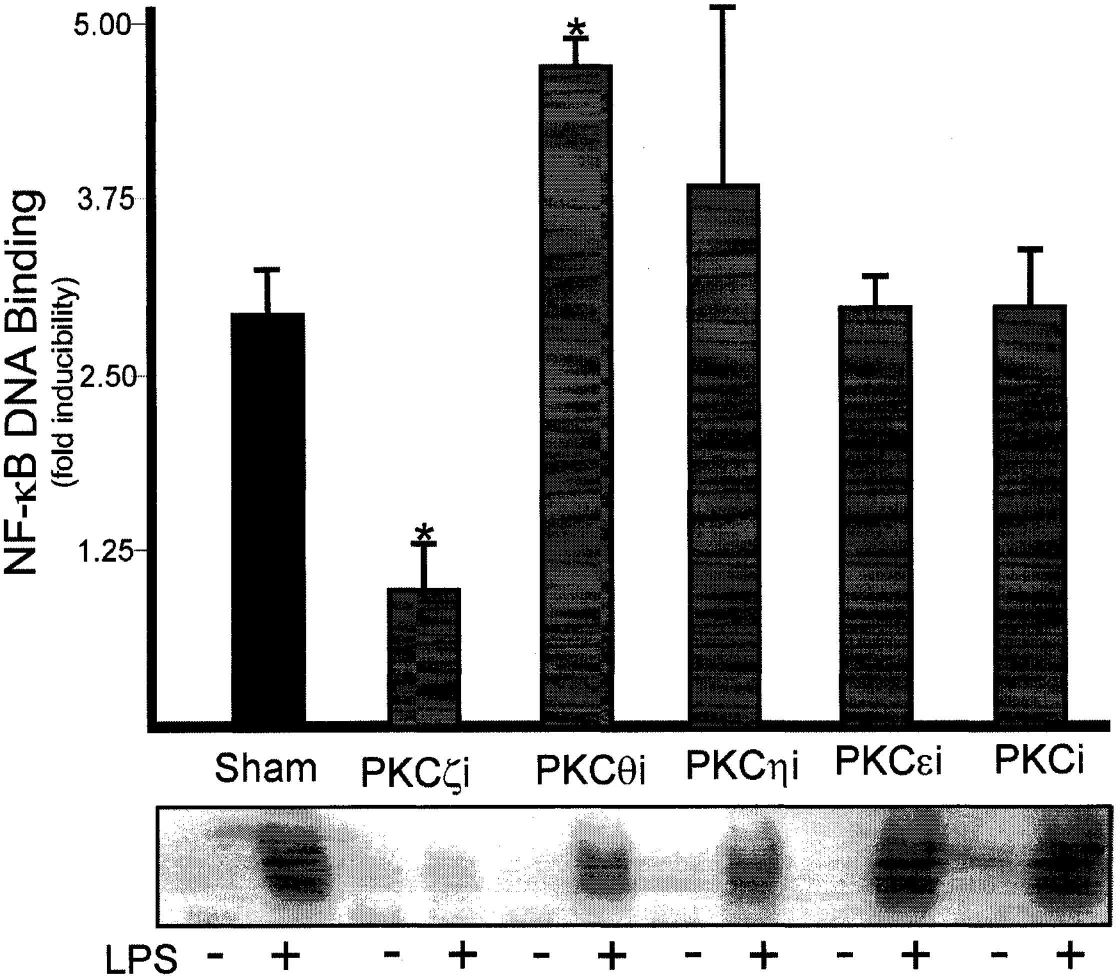

Figure 8. **Inhibition of PKCs, especially PKCζ, blocks LPS induced NF-κB DNA binding.** NRKs were pretreated with 50μM of a myrisolated pseudosubstrate peptide specific to a particular PKC or family of PKCs 30m prior to stimulation with 1μg/mL lipopolysaccharide (LPS). In each case the PKC pseudosubstrate is represented on the x-axis as the specific PKC followed by a lower case i. The pseudosubstrate used in the final 2 lanes was a pan-classical PKC inhibitor (PKCi). After LPS induction, nuclear lysates were extracted and analyzed by electromobility shift assay (EMSA) as in materials & methods. Data is presented as a fold induction of LPS induced NF-kB-DNA binding divided by the control for each pretreatment. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's t-test.

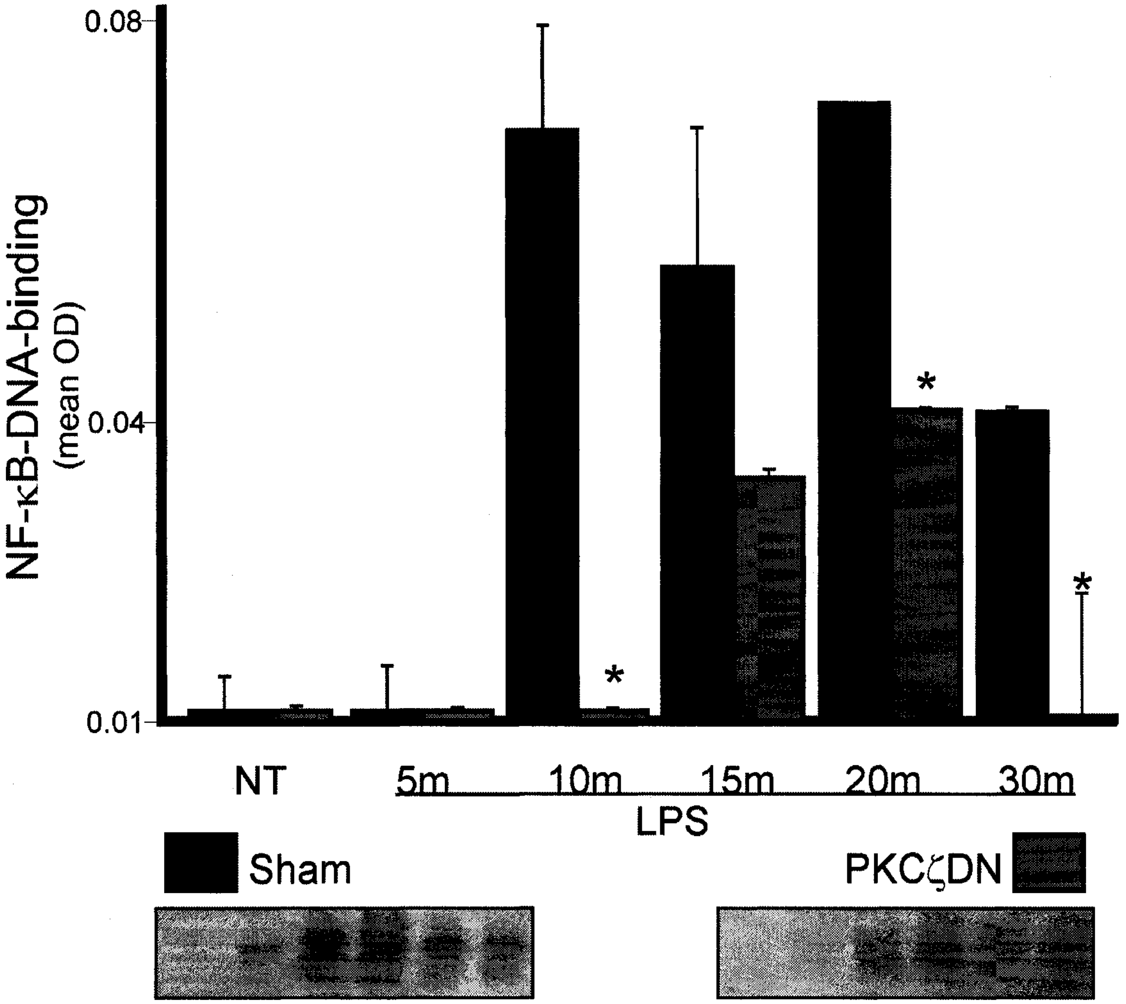

Figure 9. **PKCζ dominant negative blocks NF-κB DNA binding.** NRKs were transiently transfected with 1μg/100mm^2 surface area of a plasmid expressing a dominant negative form of PKC according to the effectene® protocol. The following morning they were stimulated with 1μg/mL LPS and harvested at the indicated times. Cells were harvested and analyzed according to the nuclear extraction and EMSA protocols, respectively, in materials and methods. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups was determined with a one-way ANOVA with Bonferroni correction using an α value of 0.05. (*)represents data points significantly different from sham.

PKCζ INHIBITION BLOCKS ACTIVATION OF IKK

From our initial experiments, we hypothesized that PKCζ regulates NF-κB upstream of the activating kinase, IKK (Figure 1). To test whether PKCζ modulates NF-κB-DNA binding through upstream kinetics, we examined IKK activity indirectly by measuring the phosphorylation status of IκBα and performed an *in vitro* IKK activity assay in PKCζDN transfected cells. We show that LPS induces the phosphorylation of IκBα maximally at 5m and that phosphorylation of this site is blocked by the expression of the PKCζDN (Fig 10). We expected the phosphorylation of IκBα to be transient, as phosphorylated IκBα is targeted for degradation [36]. In order to test IKK activity directly, we established an *in vitro* IKK activity assay based on the method of Didonato et al which measures the ability of immunoprecipitated IKK to phosphorylate a peptide corresponding to the phosphorylation region of IκBα [114]. Consistent with the lack of phosphorylation of IκBα in PKCζDN transfected cells, we show that LPS induced activation of IKK is prevented by transfection of the PKCζDN (Fig 11). Furthermore, we show that overexpression of wildtype PKCz (WTζ) increases IKK activity (Fig 11). Through these experiments, we show that PKCζ inhibition prevents the activation of IKK and the phosphorylation of the endogenous target of IKK, IκBα in a manner that is consistent with this being a mechanism through which PKCζDN inhibits NF-κB-DNA binding.

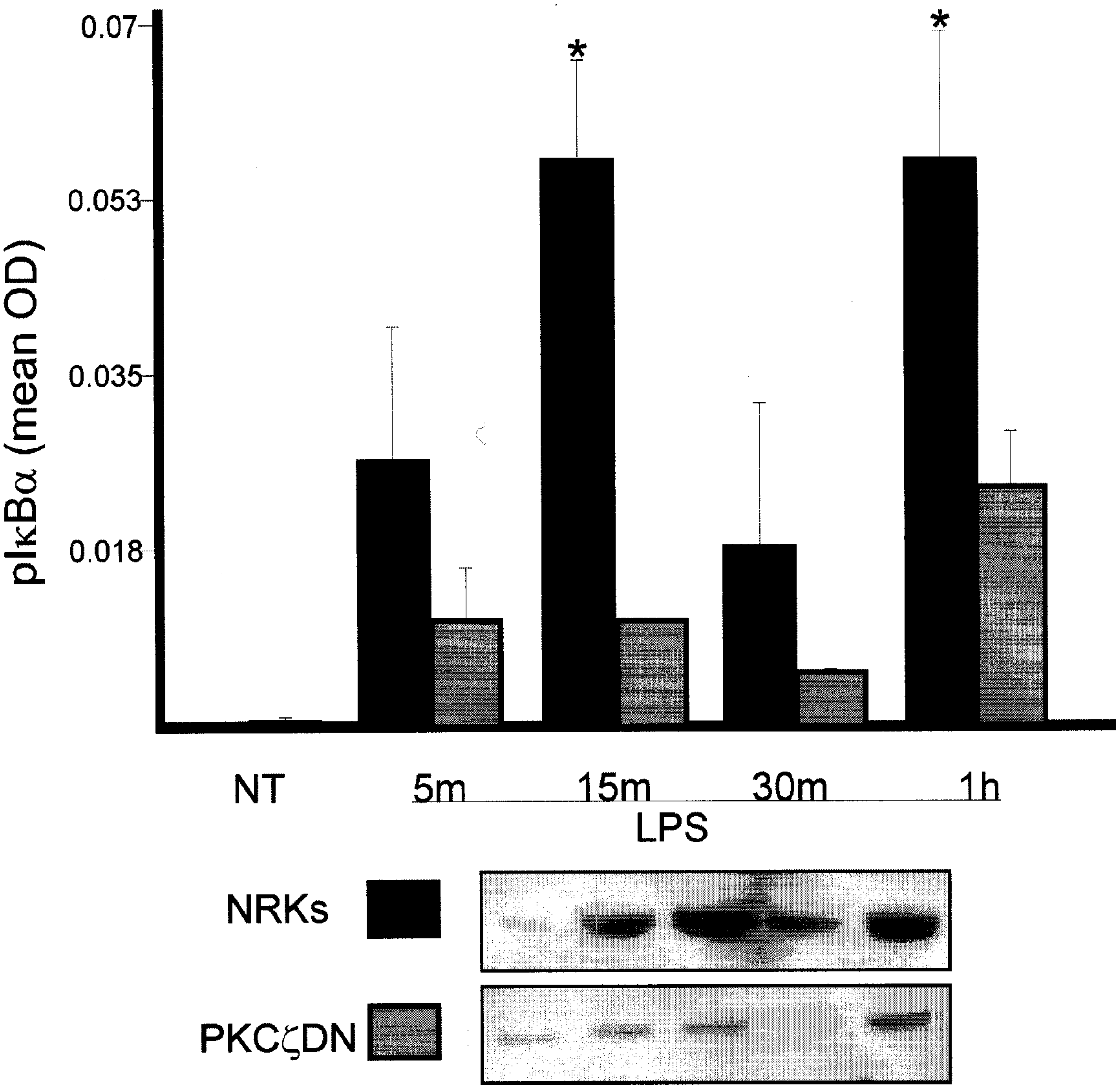

Figure 10. **PKCζDN blocks phosphorylation of IκBα at serines 32 and 36.** Cells were transfected overnight with 1μg/100mm^2 of PKCζDN. They were stimulated with 1μg/mL LPS for the time indicated and then harvested according to the western protocol in material and methods. Quantification of phosphorylation of IkBa was achieved by incubation of the membrane with an antibody that specifically recognizes phosphorylation of IκBα at these sites, followed by autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with Bonferroni correction using an α value of 0.05. (*) represents data points significantly different from sham.

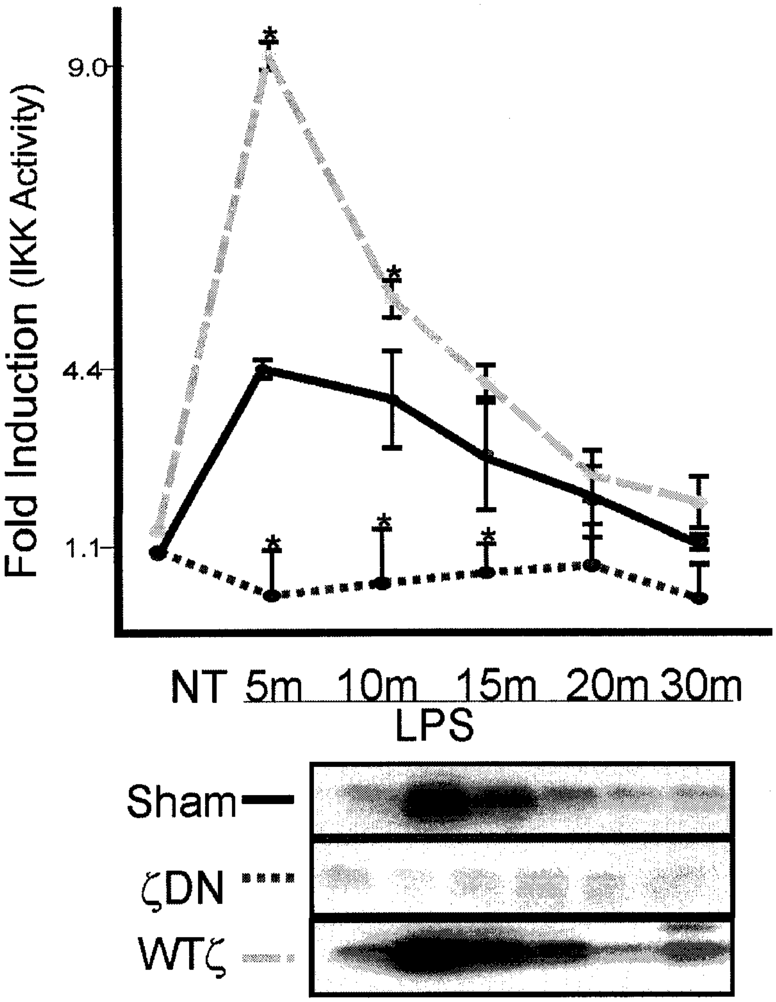

Figure 11. **PKCζDN overexpression blocks LPS induced IKK catalytic activity.** Cells were transfected overnight with 1μg/100mm^2 of either PKCζDN or wildtype PKCζ (WTζ). They were stimulated with 1μg/mL LPS for the time indicated and then harvested according to the IKKβ activity protocol in material and methods. After completion of the IKK activity assay, samples were subjected to electrophoresis and then autoradiography for quantitation. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with Bonferroni correction using an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

PKCζ AND IKK ARE COLOCALIZED AFTER LPS TREATMENT

Upon treatment with LPS, IKK and PKCζ have been shown to translocate to the plasma membrane where they are activated through interactions with upstream kinases [86, 90]. While it has been demonstrated that LPS can induce PKCζ translocation to the plasma membrane in several cells models, this has not been demonstrated to occur in kidney proximal tubule cells, which also utilize PKCζ to maintain epithelial tight junctions [99]. We show in Figure 12 that while PKCζ levels are constitutively high in the membrane fraction of these cells before LPS treatment, PKCζ is increased in the membrane fraction as early as 5m and for as long as 60m post induction with 1μg/mL LPS. The cytoplasmic expression of PKCζ was correspondingly low at all timepoints. Albeit more transient, IKK is also increased in the membrane fraction after LPS treatment (Fig 13). We found that both kinases simultaneously reach their maximal value in the membrane fraction at a time that precedes downstream NF-κB signaling, strongly implicating PKCζ as an activating kinase. We further strengthen the proposed role for PKCζ in NF-κB by the observation that immunoprecipitation with an antibody that recognizes the amino terminal end of PKCζ, contains catalytic activity toward a peptide containing the IKK phosphorylation domain of IκBα (Fig 14). Importantly, PKCζ immunoprecipitated IKK activity had the same kinetics as the IKK pulldown assay demonstrated in Figure 11. Additionally, IKKα immunoprecipitation demonstrates that PKCζ is coassociated with the signalasome in a LPS dependent manner, increasing to a maximal value at 10m post treatment (Fig 15). We demonstrate here that LPS induced colocalization of PKCζ and IKK is consistent with PKCζ acting as an upstream kinase in the NF-κB cascade in NRK cells.

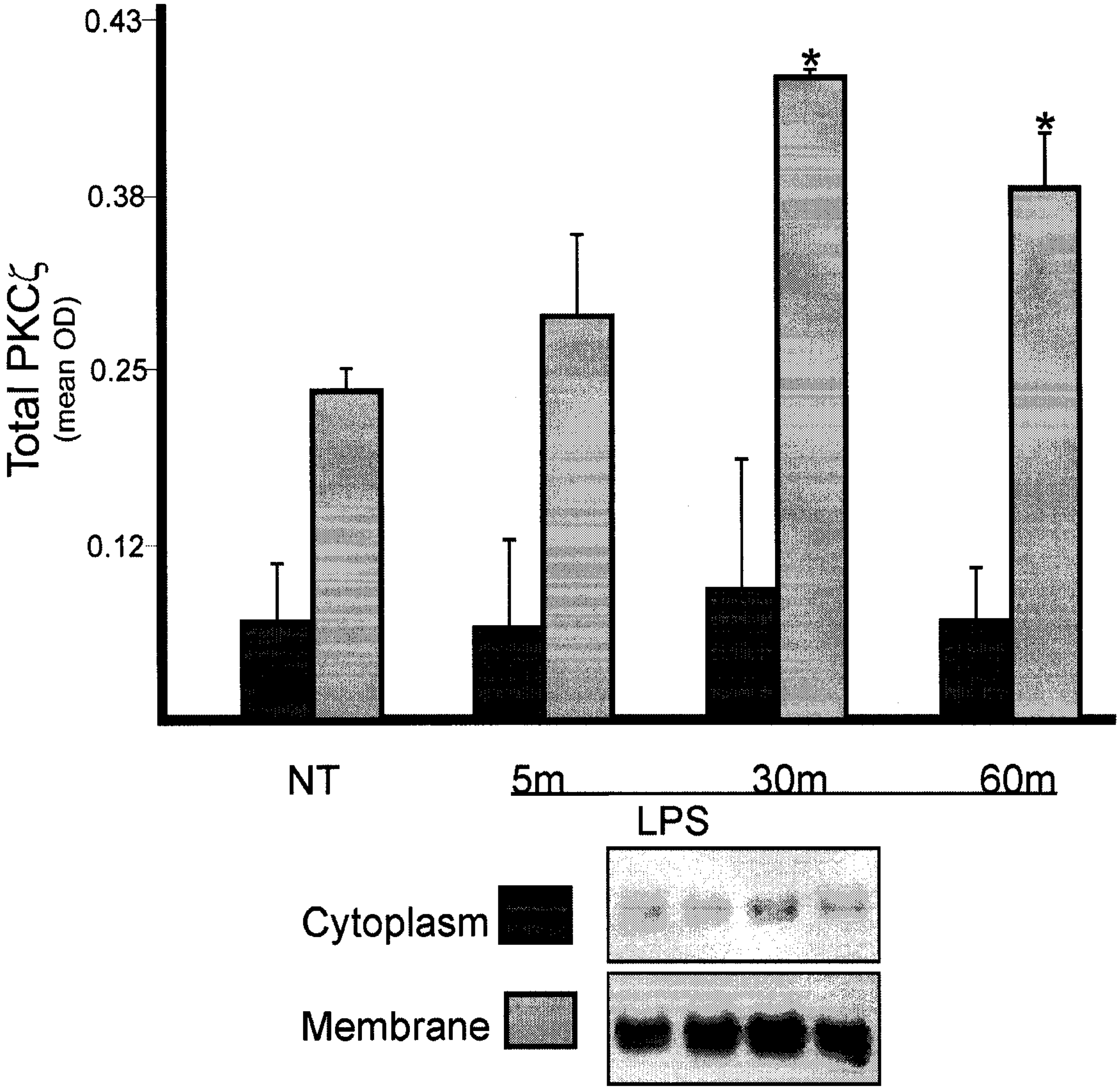

Figure 12. **PKCζ is highly enriched in the membrane fraction of NRKs, and is further increased after LPS induction.** Enriched membrane fractions were extracted from NRKs stimulated with 1μg/mL LPS for times indicated by the cellular fractionation protocol in materials. PKCζ levels were quantified by western blot followed by autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from NT (*) were determined by post hoc analysis with Dunnett's post test.

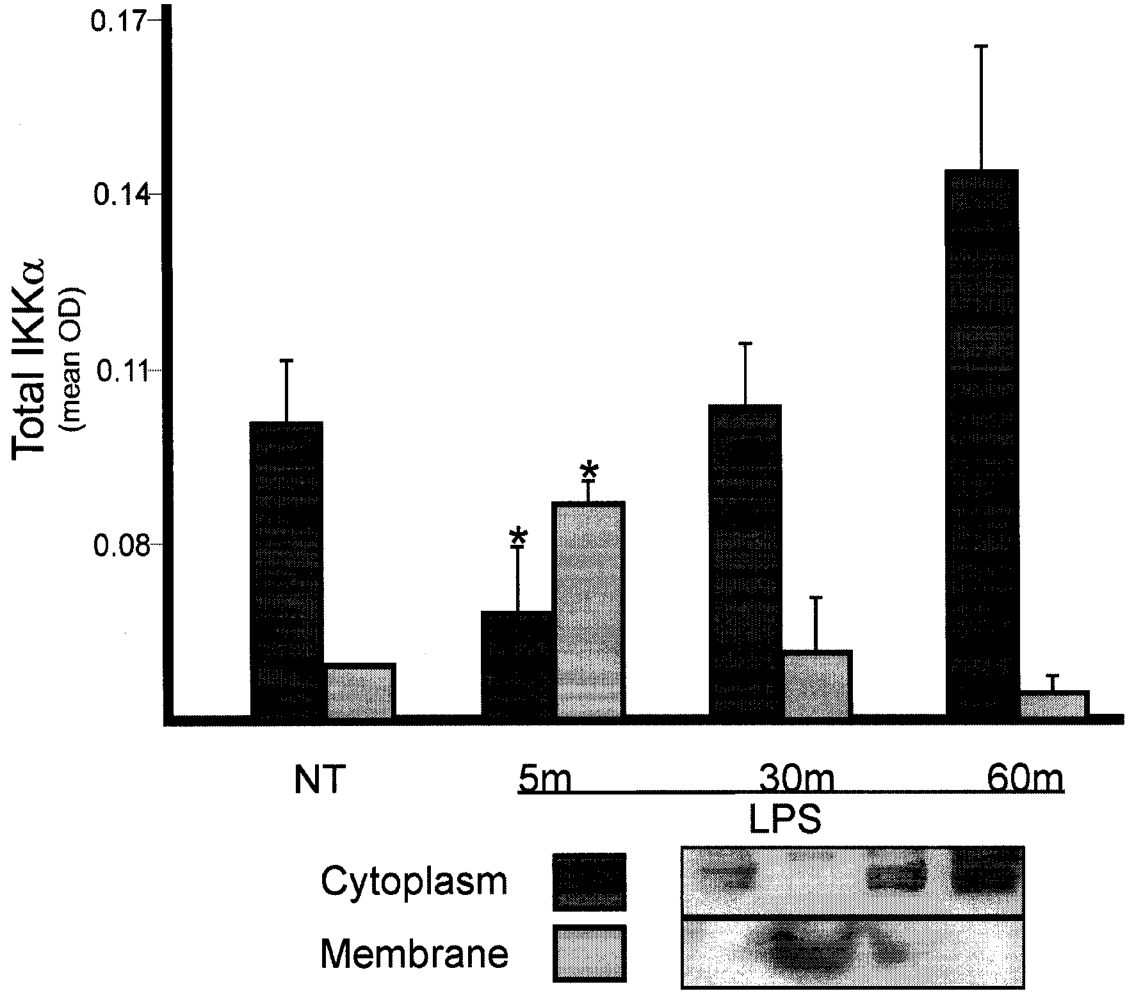

Figure 13. **The signalasome transiently increases in the membrane fraction after LPS treatment.** Enriched membrane fraction were extracted from NRKs stimulated with 1μg/mL LPS for the indicated times by the cellular fractionation assay. The signalasome concentration was determined in fractions by Western blot analysis and autoradiography using anti-IKKα. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from NT (*) were determined by post hoc analysis with Dunnett's post test.

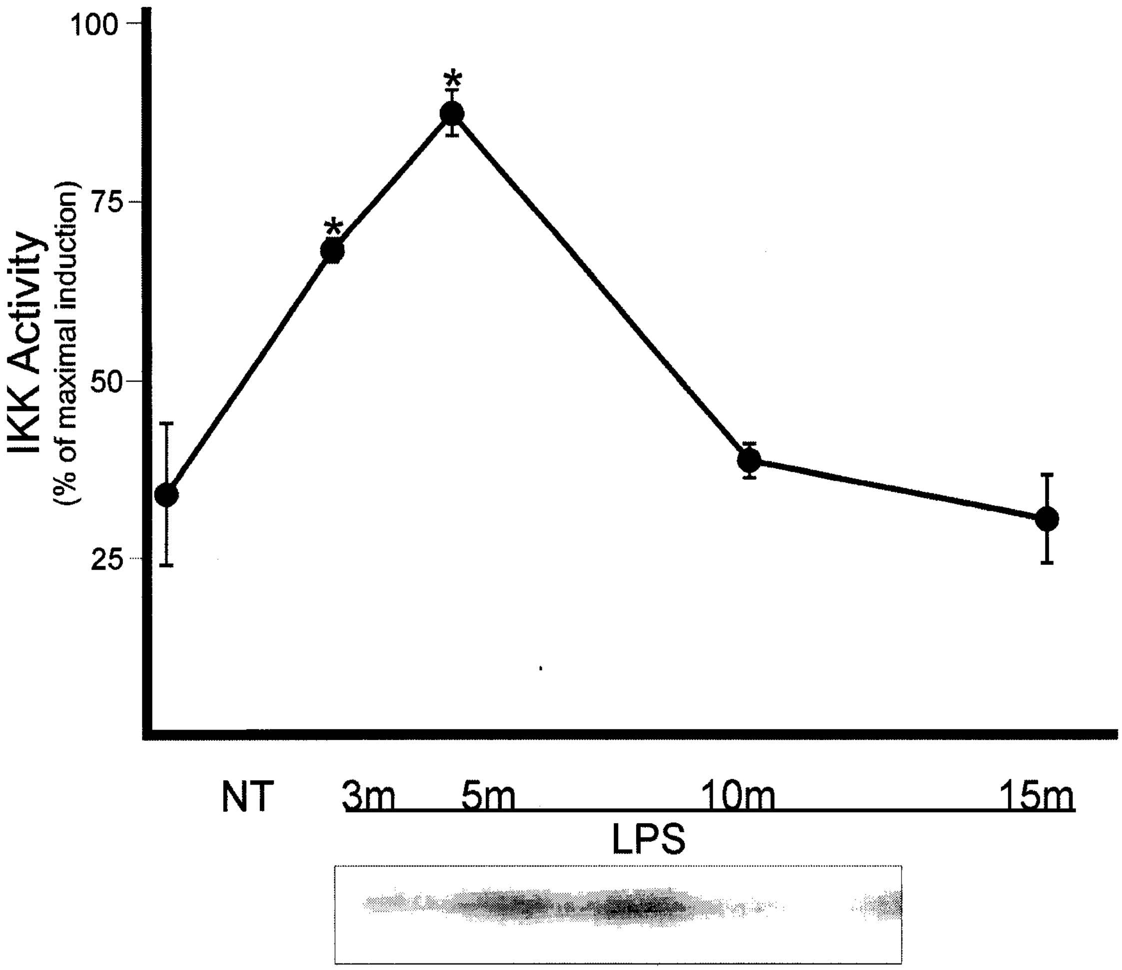

Figure 14. **PKCζ colocalizes with IKK-like activity.** NRKs induced with 1μg/mL LPS for indicated times were extracted as described in the IKK activity assay protocol. PKCζ was immunoprecipitated from 500μg total protein with 10μg of anti-PKCζ. Cleared immunoprecipitated fractions were incubated with γ-labeled P^{32}ATP and a peptide that corresponds to the IKK target region of IκBα for 10m prior to stoppage with SDS. The assay was resolved by electrophoresis and subjected to autoradiography for quantitation. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from NT (*) were determined by post hoc analysis with Dunnett's post test.

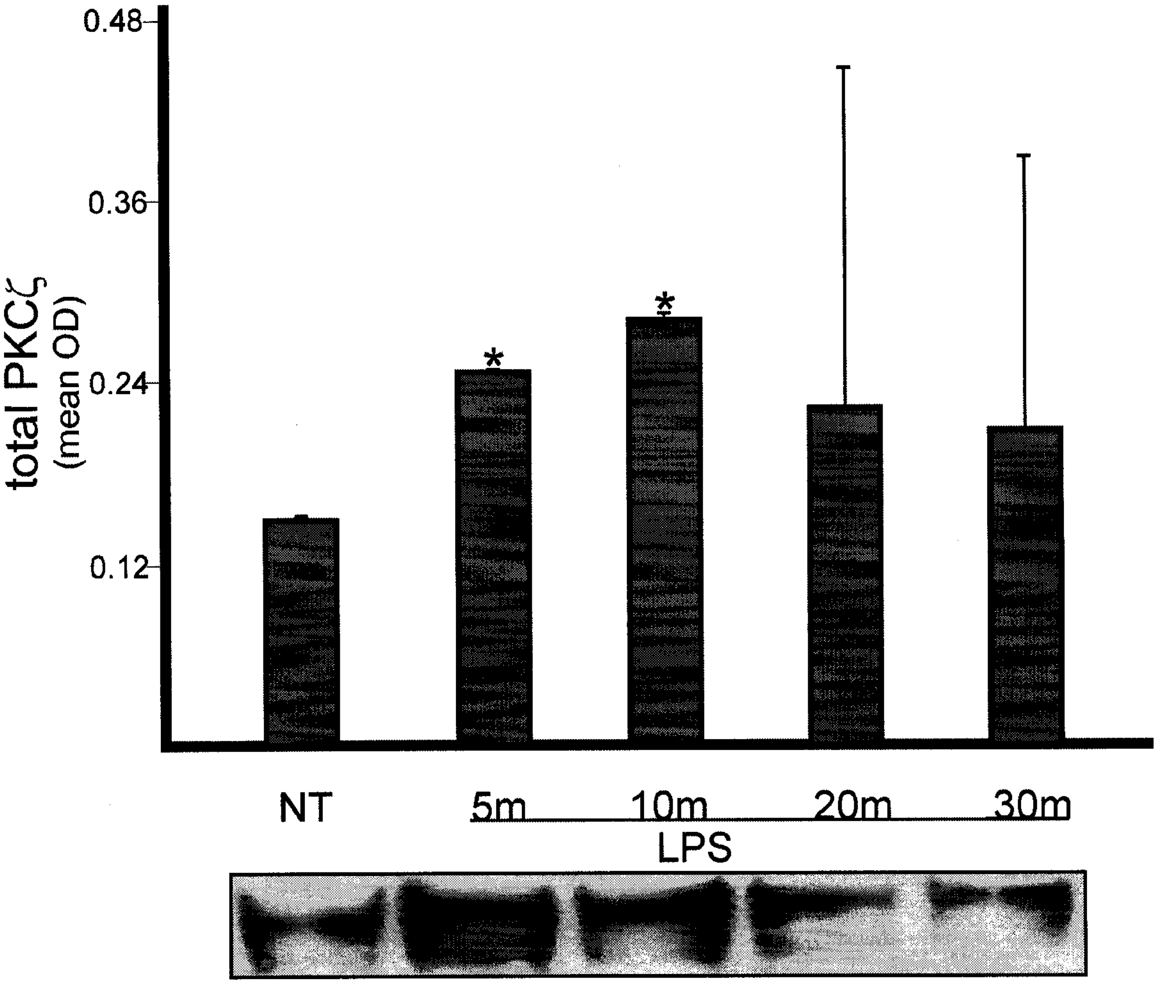

Figure 15. **IKK colocalizes with PKCζ.** Whole cell lysates were collected according to the western blotting protocol at the times indicated post LPS treatment. IKKα was IPed from 500μg total protein using 10μg of anti-IKKα. Cleared immunoprecipitated pellets were subjected to boiling in LDS loading buffer and run according to the western protocol. Quantification of PKCζ was achieved by autoradiography after probing membranes with a PKCζ antibody. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from NT (*) were determined by post hoc analysis with Dunnett's post test.

PKCζ REGULATES NF-κB TRANSACTIVATION

Once bound to the promoter of genes, NF-κB must be transactivated to initiate gene transcription [66, 67]. Transactivation involves the generation of a multi-protein complex around the NF-κB dimer, which allows for the relaxation of DNA and subsequent RNA polymerase binding associated with the recruitment of histone acetyl transferase activity [36]. PKCζ is an activator of NF-κB transactivation and is required for the expression of several NF-κB driven genes after cytokine treatment [72]. We demonstrate that inhibition of PKCζ with the myrisolated PKCζ pseudosubstrate reduces LPS mediated NF-κB transactivation, prevents LPS activation at 2h and reduces it by 50% at 4h post treatment (Fig 16). Furthermore, we show that inhibition of PKCζ by the transient transfection of the PKCζDN blocks the LPS induced NF-κB transactivation at 4h (Fig 17). Surprisingly, transient transfection of wildtype PKCζ (WTζ) also blocks NF-κB transactivation (Fig 17). While we find the inhibition of NF-κB transactivation by PKCζ unexpected, PKCζ is known to impact several other pathways (notably AKT) which are responsible for NF-κB transactivation. Considering that in other models PKCζ inhibition can block NF-κB transactivation even when it does not block IKK activation, we decided to test the ability of the stably transfected PKCζDN and WTζ to confirm the transient transfection findings [112]. As transfection introduces an additional confounding condition, we tested whether stable transfection of PKCζDN would confirm the findings obtained with the transient expression of PKCζDN. Indeed, we find that transient expression of PKCζDN blocks NF-κB transactivation (Fig 18). Additionally we confirmed the finding that WTζ is inhibitory towards NF-κB transactivation (Fig 18). The luciferase construct used in Figures 16, 17 and 18 measures NF-κB transactivation induced by p65 binding to a 4x tandem repeat domain, which then activates downstream expression of luciferase. The drawback of this system is that it is dependent on NF-κB DNA-binding as well as transactivation for expression, to overcome this complication; we used a fusion reporter system in which a β-galactosidase/p65 fusion binds to a gal-4/luciferase construct. This system offers the advantage of being independent of NF-κB DNA binding, and measures p65 transactivation but not transactivation based on other components of NF-κB [10]. We demonstrate that both PKCζDN and WTζ inhibit p65 transactivation directly when stably transfected, independent of NF-κB DNA-binding (Fig 19). Through these experiments, we demonstrate that PKCζ can regulate transactivation of p65 independently of NF-κB DNA-binding.

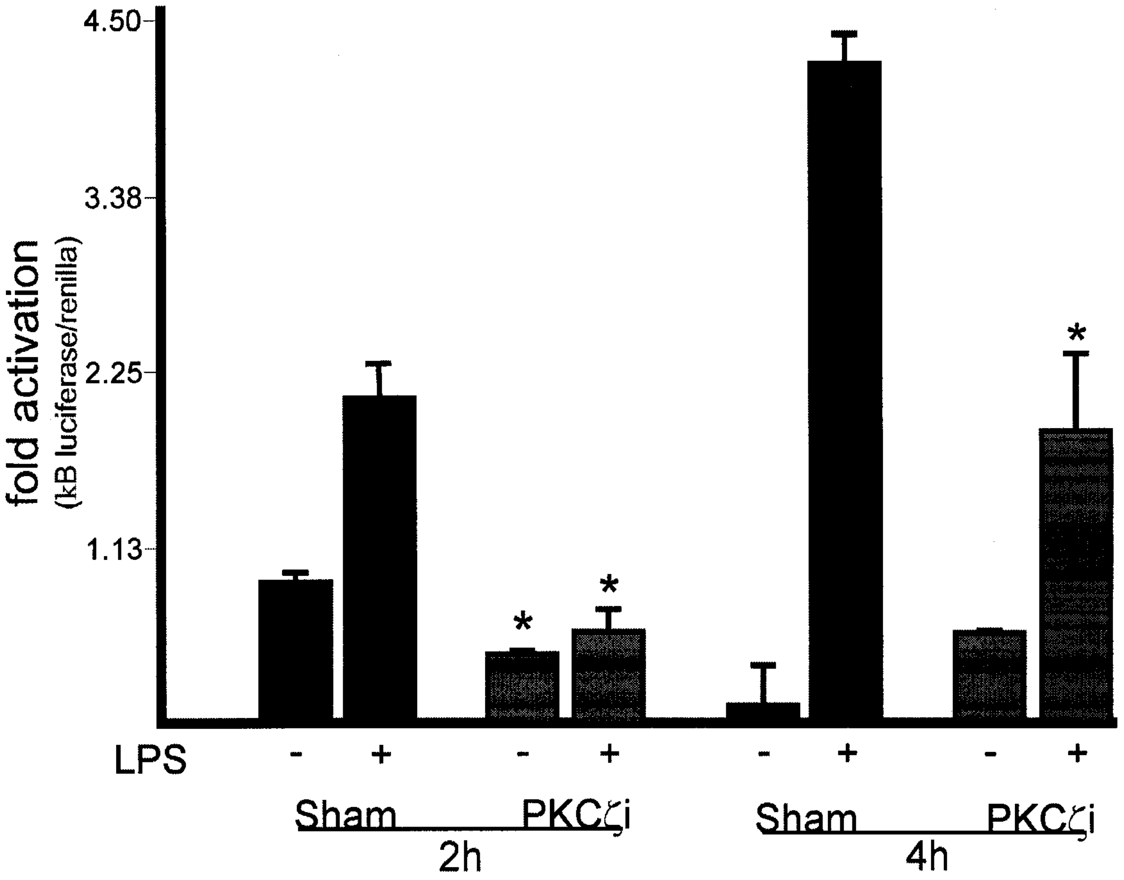

Figure 16. **PKCζ pseudosubstrate reduces NF-κB transactivation.** NRKs were transfected overnight with 400ng/well of 4x-luciferase and 20ng/well CMV driven renilla. The cells were harvested in passive lysis and analyzed by luminescence according to Promega's protocol after 2 or 4h stimulation with 1μg/mL LPS stimulation. Pretreatment with the PKCζ pseudosubstrate (PKCζi) was carried out for 30m prior to LPS treatment. These data are represented as fold activation of the 2h constitutive level of luciferase after correction for transfection efficiency with the renilla. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a two-tailed *t*-test using an α value of 0.05 and bonferroni correction. *represents significantly different from corresponding sham samples.

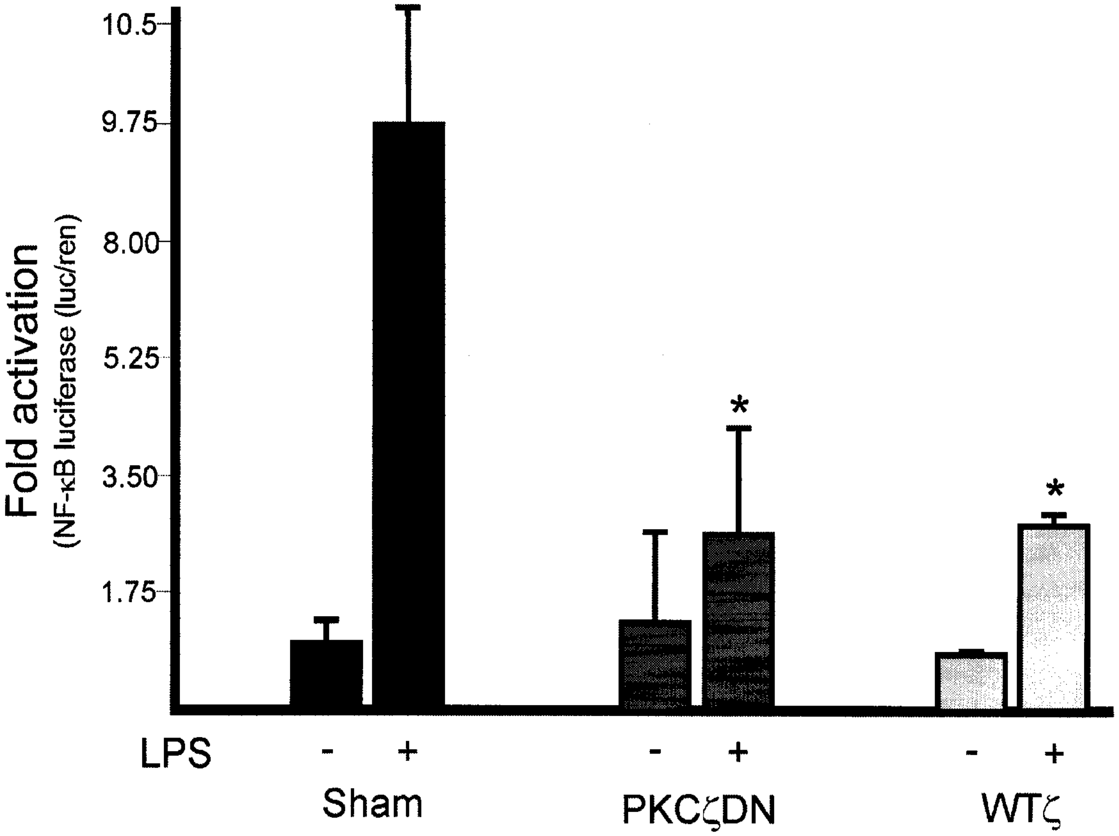

Figure 17. **PKCζ alteration blocks transactivation.** NRKs were transiently cotransfected overnight with 400ng/well of 4x-luciferase and 20ng/well CMV driven Renilla luciferase and either 1μg/100mm^2 dominant negative or wildtype PKCζ (PKCζDN and WTζ, respectively). The cells were harvested in passive lysis and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS. These data are represented as fold activation of the sham constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from corresponding NT (*) were determined by post hoc analysis with Dunnett's post test.

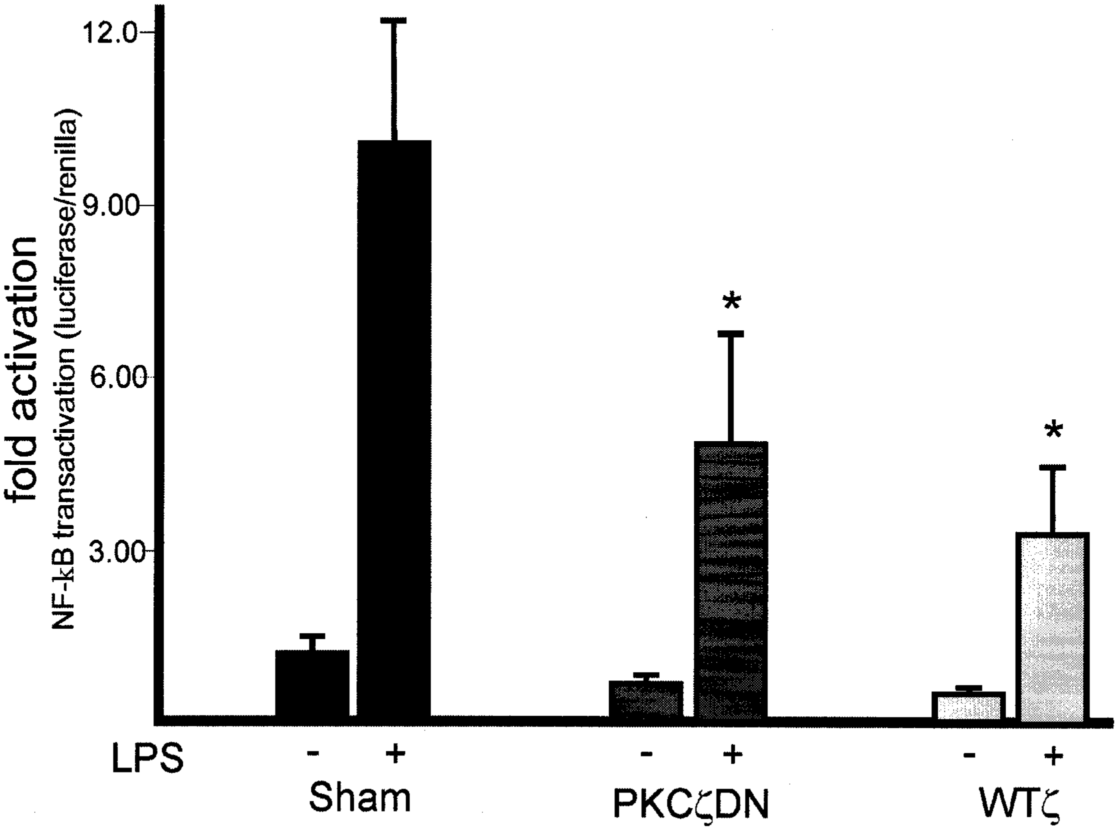

Figure 18. **Stable overexpression of PKCζ inhibits transactivation, irrespective of catalytic activity.** NRKs stably expressing PKCζDN or PKCζ were transiently transfected overnight with 4x-luciferase prior to 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS. These data are represented as fold activation of the sham transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from corresponding sham (*) were determined by post hoc analysis with Dunnett's post test.

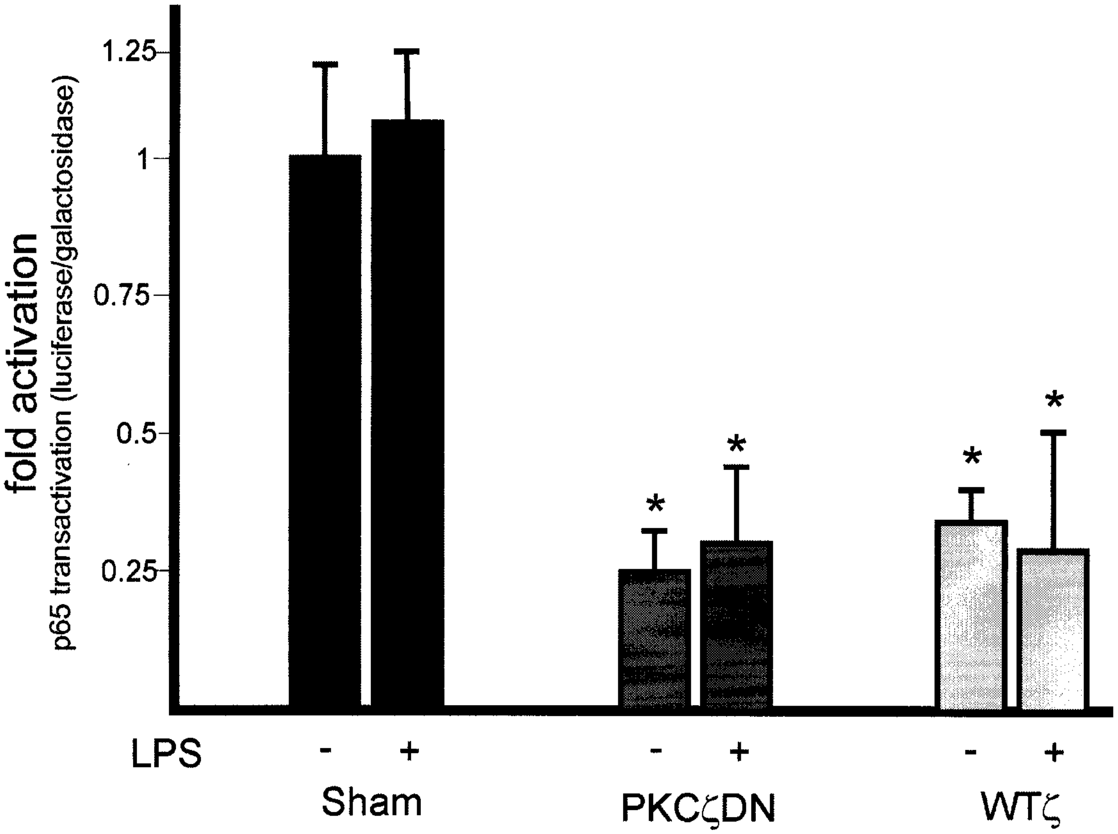

Figure 19. **Stable overexpression of PKCζ blocks transactivation independent of translocation.** NRK cells stably overexpressing PKCζDN or WTζ were transfected overnight with 100ng/well of gal4 luciferase and 25ng/well of a vector expressing a gal4/p65 fusion protein before being stimulated with LPS for 4h. The cells were harvested in Passive Lysis Buffer and then split for luciferase and galactosidase analysis. Luciferase analysis was carried out with the Dual luc kit and galactosidase activity was carried out according to the β-galactosidase enzyme assay, according to Promega's protocol. These data are represented as fold activation of the sham constitutive level of luciferase after correction for transfection efficiency with galactosidase activity. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from corresponding sham (*) were determined by post hoc analysis with Dunnett's post test.

PKCζ REGULATES THE HAT ACTIVITY ASSOCIATED WITH p65 THROUGH MULTIPLE MECHANISMS

Having determined that both PKCζDN and WTζ inhibit LPS mediated NF-κB transactivation in these cells, we sought to better define the mechanism of this inhibition. We hypothesized that stably transfected PKCζDN blocks the interaction of p65 with p300. Histone acetyl transferases (HATs) like p300 must be recruited to sites of active transcription to relieve histone induced supercoiling, which otherwise prevents processive RNA polymerase activity and gene induction (Figure 5) [115]. To test whether PKCζ prevents p65 from interacting with p300, we established an immunoprecipitation protocol for p300, in which we show that LPS induced colocalization of p65 with p300 is blocked by expression of PKCζDN (Fig 20). Furthermore, we show that overexpression of active PKCζ (WTζ) potentiates the ability of p300 to colocalize with p65 (Fig 20). And although PKCζ overexpression increases the colocalization of p65/p300, we show that global p300 activity, as measured by a p300/galactosidase reporter system, is decreased in WTζ transfected cells, thus limiting the potential HAT activity capable of associating with p65 (Fig 21). Notably, we found that LPS does not induce p300 activity in these cells. We demonstrate that PKCζ is necessary for the interaction of p65 with the transactivation activator p300, but that overexpression of PKCζ limits the amount of HAT activity associated with p65 by directly inhibiting p300 activity.

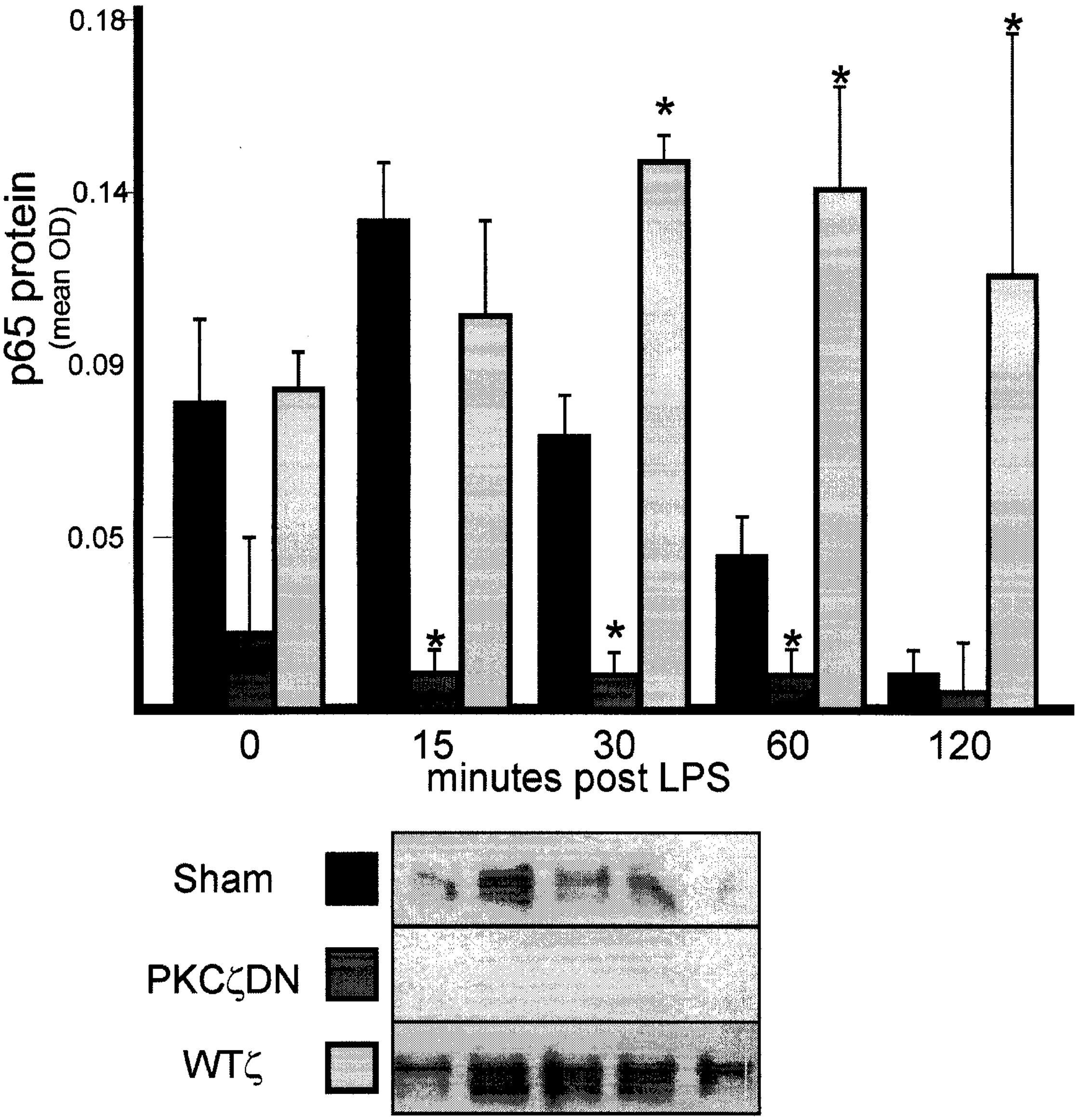

Figure 20. **p300 colocalization with p65 is enhanced by PKCζ overexpression.** Whole cell lysates were taken from NRKs stably expressing empty vector (sham), a dominant negative PKCζ (PKCζDN) or wildtype PKCζ (WTζ). p300 was immunoprecipitated with 1µg anti-p300 from 500µg total protein for 3h as in material and methods. Cleared fractions were subjected to Western blot analysis and audioradiography for quantification of p65 bound to p300. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

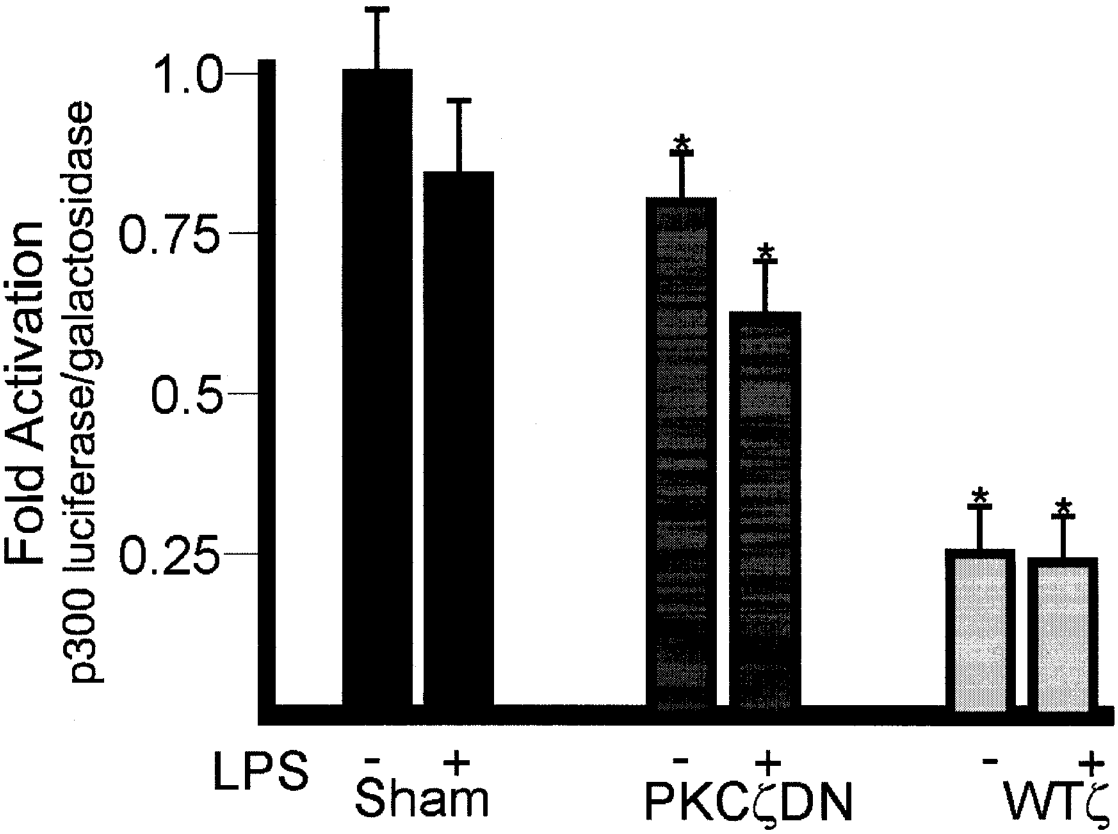

Figure 21. **PKCζ overexpression decreases the activity of a p300/gal4 reporter system.** NRKs stably expressing PKCζDN or WTζ were transiently transfected overnight with 100ng/well of gal4 luciferase and 25ng/well of a vector expressing a gal4/p300 fusion protein before being stimulated with LPS for 4h. The cells were harvested in passive lysis and analyzed for luminescence and galactosidase activity according to the appropriate Promega protocol, as in materials and methods. These data are represented as fold activation of the sham transfected constitutive level of luciferase after correction for transfection efficiency with the galactosidase acitivity value. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

PHOSPHORYLATION OF p65 AT SERINE 311 IS MODULATED BY PKCζ, BUT IS NOT THE SITE OF TRANSACTIVATION INHIBITION

PKCζ can regulate p65/p300 colocalization through the phosphorylation of p65 on serine 311 [72]. Using a mutant form of p65 that is incapable of being phosphorylated at serine 311, due to a mutation of this site to alanine (S311A), we show that blockage of serine 311 phosphorylation reduces the ability of a p65 construct to induce NF-κB transactivation, but only slightly (Fig 22). Furthermore, we determined that stable transfection of PKCζDN did not block the phosphorylation of serine 311 (Fig 23). Consistent with previous reports however, we find that PKCζ does impact this site, as stable overexpression of PKCζ (WTζ) increases the phosphorylation of p65 at this site (Fig 23). However, we did not find that inhibition of PKCζ by stable overexpression PKCζDN blocks phosphorylation of p65 at serine 311 (Fig 23). We are unsure if the lack of ability of stably transfected PKCζDN to block serine 311 phosphorylation is a result of incomplete blockage of constitutive PKCζ activity or if another kinase is capable of compensating for PKCζ in these cells. Considering that the role of serine 311 phosphorylation has been delineated, we focused our efforts to determine how the PKCζDN was blocking NF-κB transactivation. We demonstrate that serine 311 is not the site of action of the PKCζDN as it does not block phosphorylation of p65 at this site.

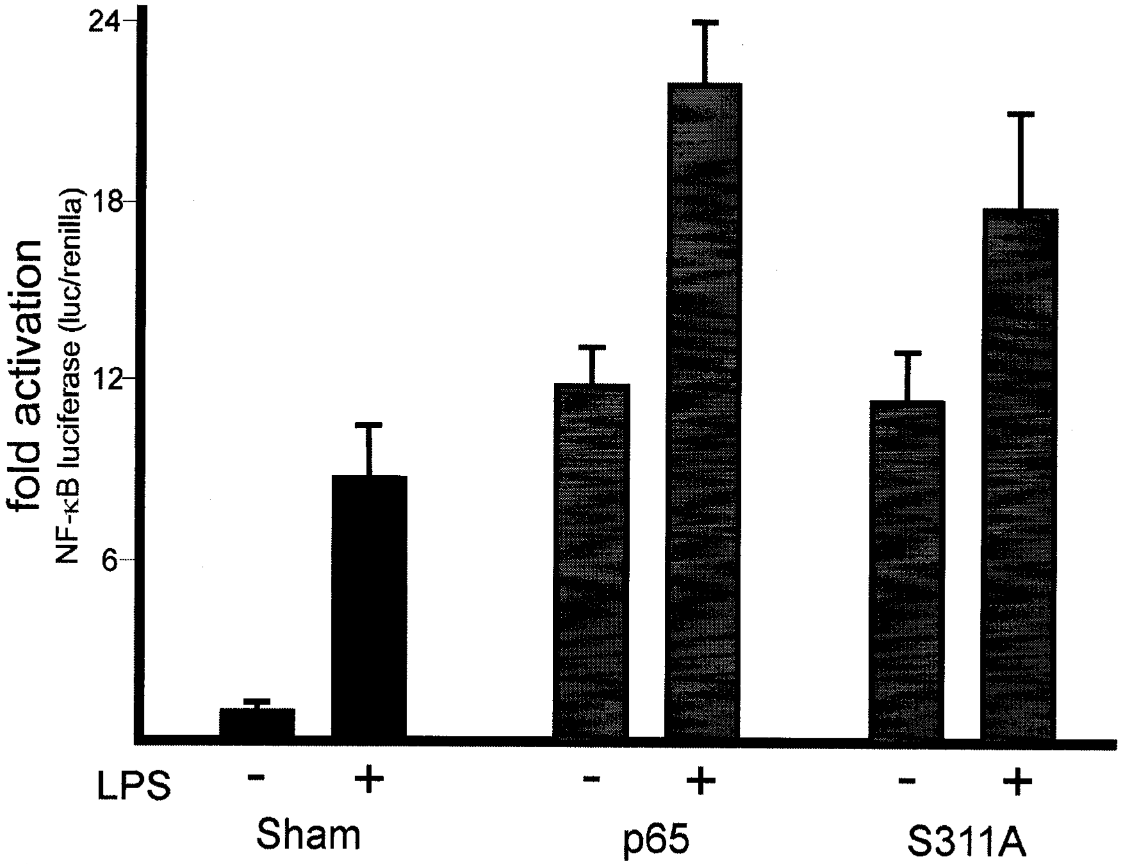

Figure 22. **Transactivation is slightly inhibited by the mutation of p65 at serine 311.** NRK cells were transiently cotransfected overnight with 400ng/well of 4x-luciferase and 20ng/well CMV driven Renilla luciferase and 1μg/100mm^2 of an expression vector containing wildtype 65 or p65 with serine 311 mutated to alanine (S311A). The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS. These data are represented as fold activation of the sham constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from p65 (*) were determined by post hoc analysis with Dunnett's post test.

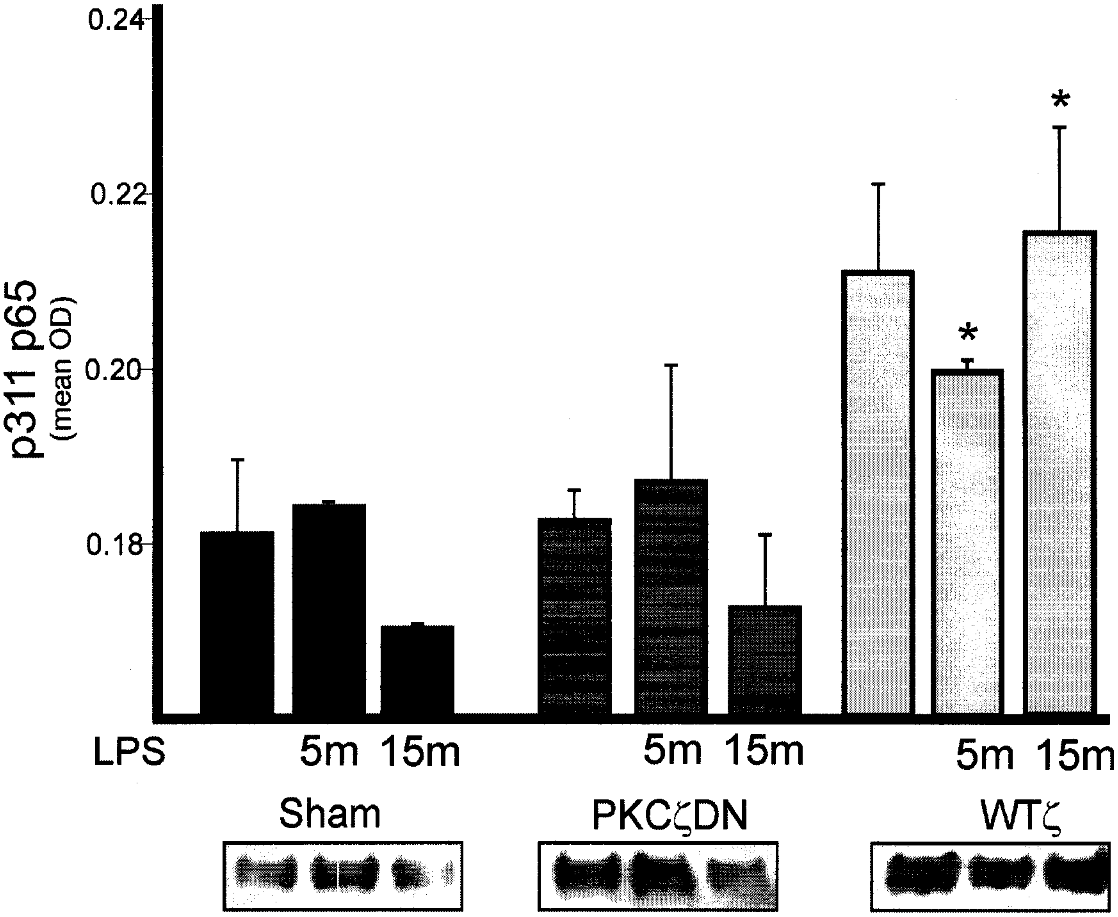

Figure 23. **PKCζ overexpression increases phosphorylation of p65 at serine 311.** Whole cell lysates were taken from NRK cells stably overexpressing empty vector, dominant negative PKCζ (PKCζDN), or wildtype PKCζ (WTζ) at indicated times post stimulation with 1μg/mL LPS. The lysates were normalized for total protein using the Biorad protein assay and p65 was immunoprecipitated as in materials and methods with 1μg of p65 antibody. The purified fraction was subjected to Western blot analysis and membranes were probed with anti phospho 311 p65 prior to autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

PHOSPHORYLATION OF p65 ON SERINE 536 IS CRITICAL FOR TRANSACTIVATION, BUT IS PKCζ INDEPENDENT

NF-κB transactivation is regulated by multiple modifications [36]. IKK mediated phosphorylation of p65 on serine 536 has been reported as critical for the transactivation of NF-κB. Based on the ability of PKCζDN to attenuate IKK activity (Fig 11), we hypothesized that PKCζDN blocks NF-κB transactivation by preventing IKK mediated phosphorylation of p65 on serine 536. Using a mutant form of p65 that can not be phosphorylated at serine 536 due to a single base change from serine to alanine (S536A), we tested the necessity of phosphorylation of serine 536 on NF-κB transactivation. We found that S536A p65, as compared to overexpression of wildtype p65, is much less capable of facilitating NF-κB transactivation after LPS treatment, and that this inhibition is so complete that the maximal transactivation response in cells expressing S536A p65 is no greater than that seen in sham transfected cells (Fig 24). We attribute the higher background of transactivation in S536A transfected cells to the competition of S536A p65 with wildtype p65 for IκBα binding, resulting in a release of endogenous pools of p65. To test the role of serine 536 without the need for overexpression, we confirmed that IKK mediates phosphorylation of serine 536 in these cells by showing that the IKK inhibitors BMS-345531 (BMS) and Wedlolactone (Wedlo) prevent LPS induced phosphorylation of p65 on serine 536 (Fig 25). We confirmed that the IKK inhibitors block NF-κB transactivation as measured by the 4x luciferase reporter system (Fig 26). However the results from this system are confounded by the necessity of NF-κB translocation prior to activation of this reporter. In the p65/galactosidase reporter system, we were unable to demonstrate that the IKK inhibitor BMS-345531 has a significant inhibitory effect on the transactivation of p65 at the doses used to block phosphorylation of p65 on serine 536 (Fig 27). The discrepancy between these systems likely arises from the dependence of the NF-κB luciferase on IKK-dependent translocation, and the independence of the p65/galactoside system on translocation. As IKK inhibition does not block p65 transactivation but stable expression of PKCζDN and WTζ do, we felt it unlikely that stable expression of PKCζDN or WTζ impacted NF-κB transactivation through IKK mediated phosphorylation of p65 on serine 536. We confirmed that stable expression of neither PKCζDN nor WTζ prevents the phosphorylation of p65 at serine 536 (Fig 28). We are unclear as to how PKCζDN inhibits IKK dependent translocation of p65 but not IKK dependent phosphorylation of serine 536, although PKCζ could regulate the target specificity of the signalasome by changing the binding partners of the signalosome through a similar mechanism as demonstrated for PKCθ [115]. By overlaying the IKK activity timecourse from Figure 4, we demonstrate that a temporal relationship exists between IKK and serine 536 phosphorylation (Fig 28). We demonstrate that stable expression of PKCζDN does not block NF-κB transactivation through modulation of p65 at serine 536.

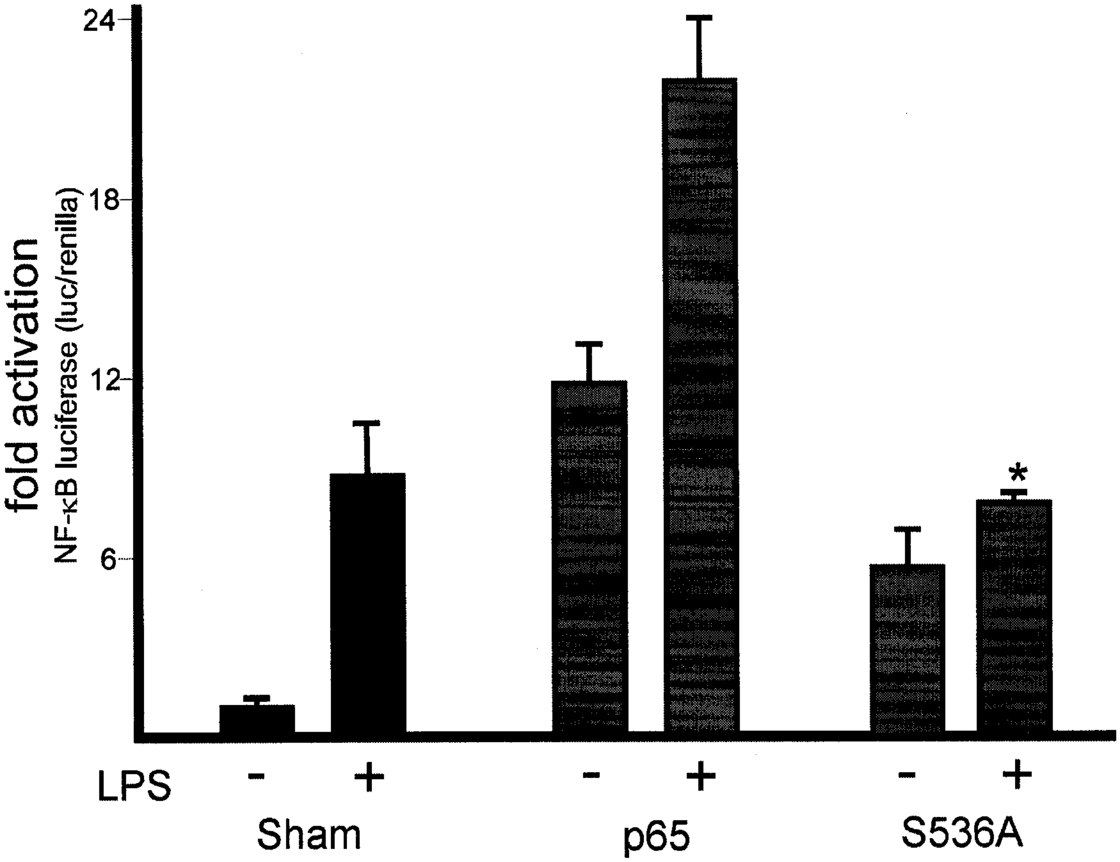

Figure 24. **Transactivation is inhibited by the mutation of p65 at serine 536.** NRKs were transiently cotransfected overnight with 400ng/well of 4x-luciferase and 20ng/well CMV driven Renilla luciferase and 1μg/100mm^2 of an expression vector containing wildtype p65 or p65 with serine 536 mutated to alanine (S536A). The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS stimulation. These findings are represented as fold activation of the sham constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. The sham and p65 transfected data are the same as displayed in Figure 25. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from p65 (*) were determined by post hoc analysis with Dunnett's post test.

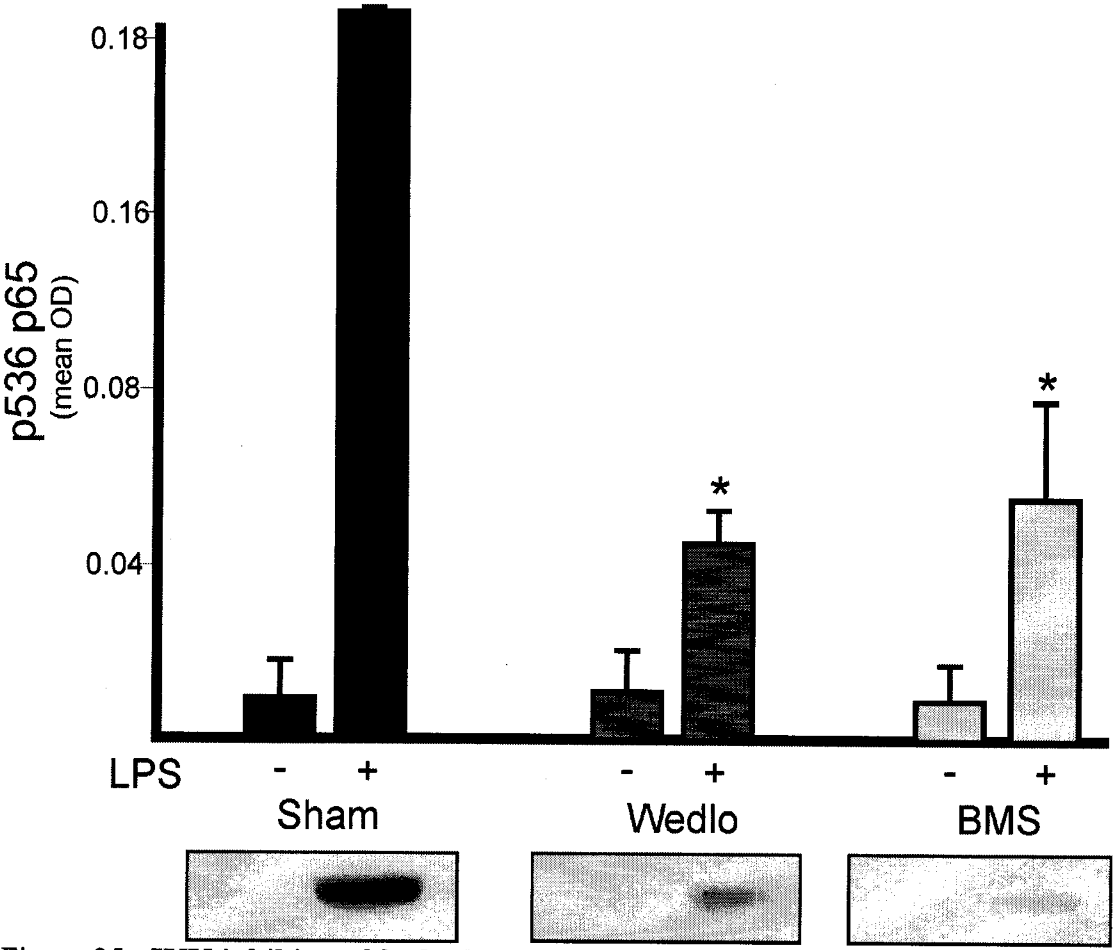

Figure 25. **IKK inhibitors block phosphorylation of p65 at serine 536.** Whole cell lysates were taken from NRK cells at the indicated times post stimulation with 1μg/mL LPS. Pretreatment with 30μM of the IKK inhibitors wedlolactone (wedlo) or BMS-345531 (BMS) was carried out for 30m prior to LPS treatment. The lysates were normalized for total protein using the biorad protein assay and were subjected to Western blot analysis. Membranes were probed with phospho 536 p65 antibody prior to autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

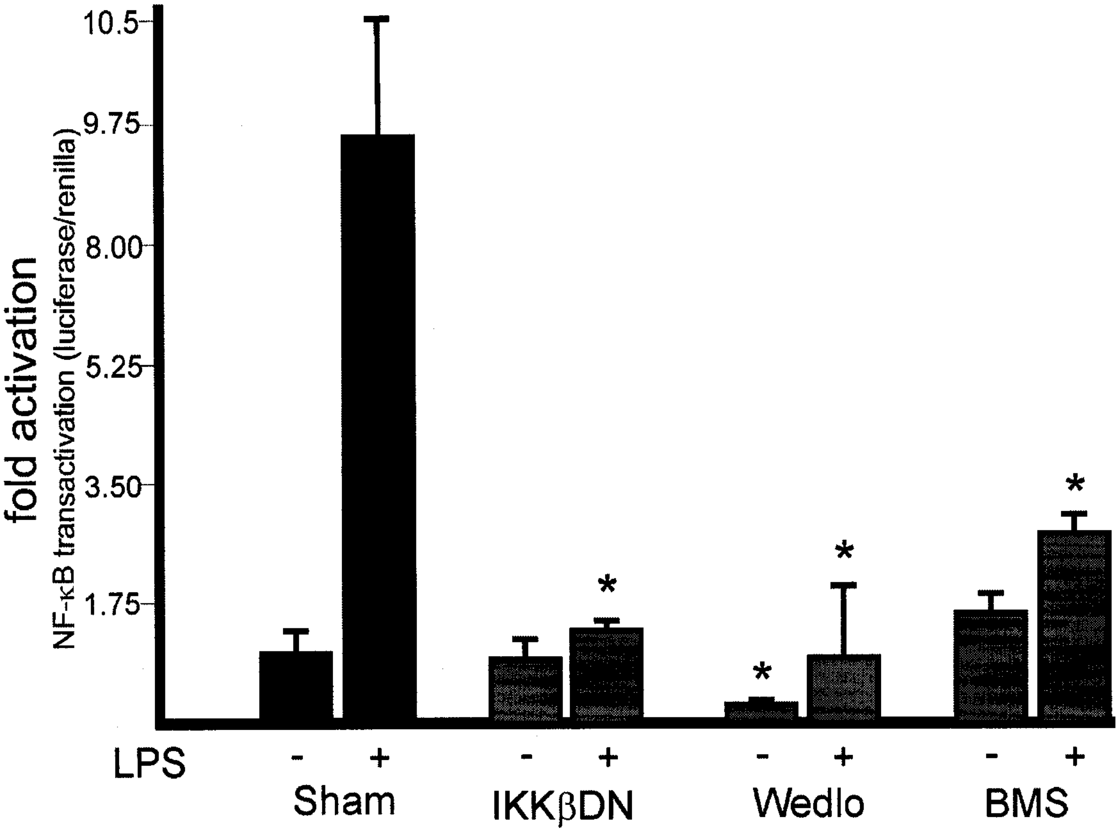

Figure 26. **IKK inhibitors block NF-κB transactivation.** NRK cells were transiently transfected overnight with 400ng/well of 4x-luciferase and 20ng/well CMV driven Renilla luciferase, as well a 1μg/mm^2 of a dominant negative to IKKβ (IKKβDN) in indicated samples. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS. These findings are represented as fold activation of the sham transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

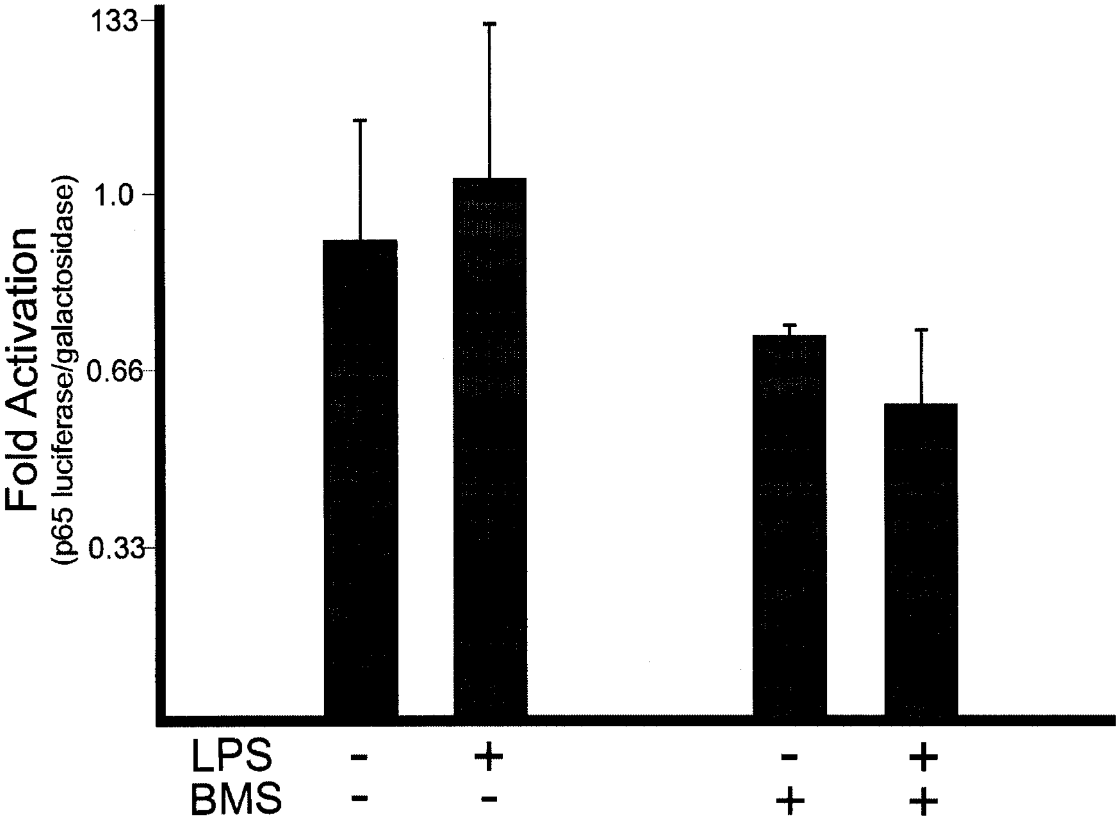

Figure 27. **IKK inhibition do not significantly alter the transactivation of the p65/ galactosidase fusion protein.** NRK cells were transfected overnight with 100ng/well of gal4 luciferase and 25ng/well of a vector expressing a gal4/p65 fusion protein before being pretreated for 1h with BMS-345531 (BMS) and stimulated with LPS for 4h. The cells were harvested in Passive Lysis Buffer and then split for luciferase and galactosidase analysis. Luciferase analysis was carried out with the Dual luc kit and galactosidase activity was carried out with the b-galactosidase enzyme assay, according to Promega's protocol. Data shown are means ± SD of at least 3 replicate experiments. No difference between treatment groups were observed by a two tailed t test with an α of 0.05.

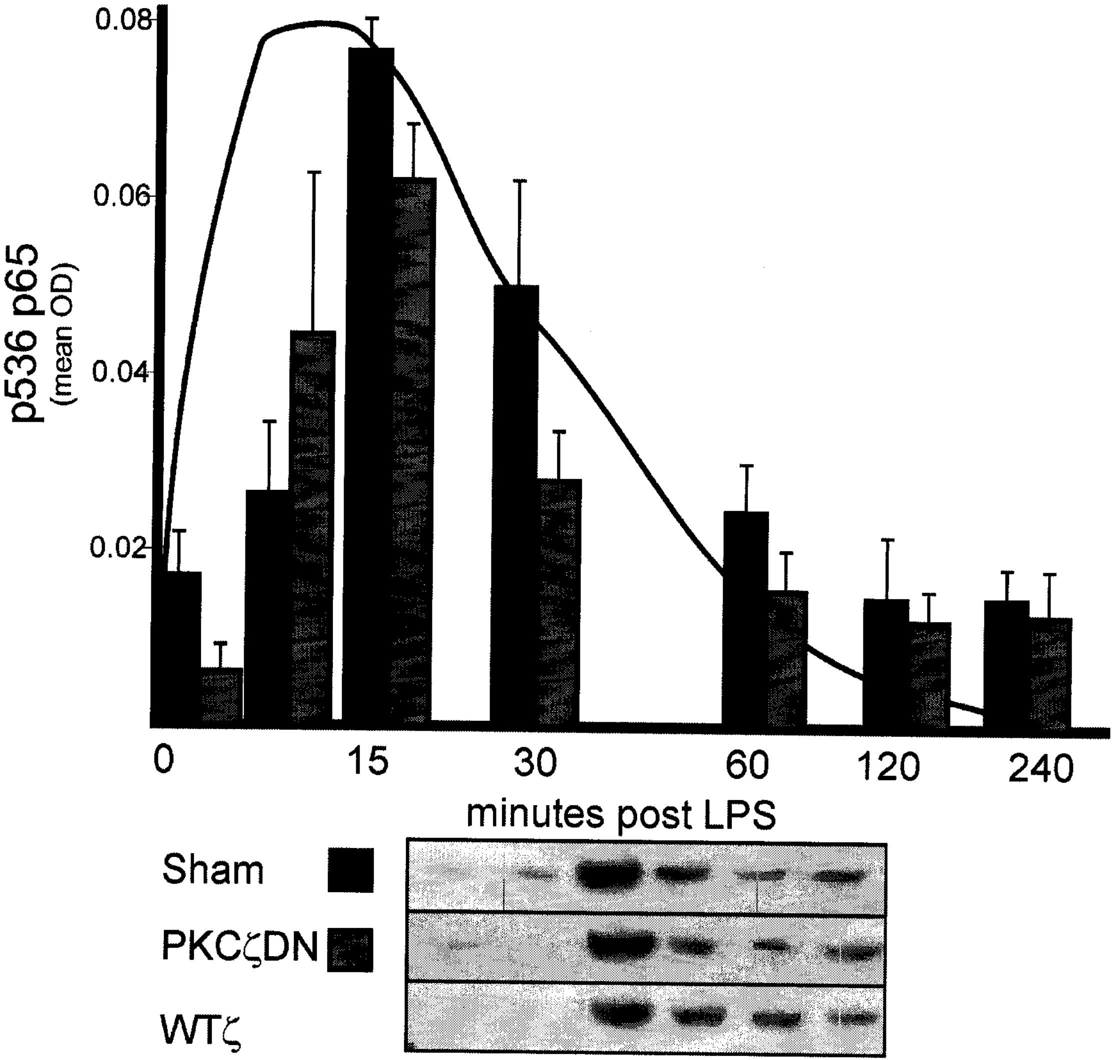

Figure 28. **Phosphorylation of p65 at Serine 536 is independent of PKCζ.** Whole cell lysates were taken from NRK cells stably overexpressing empty vector, dominant negative PKCζ (PKCζDN), or wildtype PKCζ (WTζ) at the indicated times post stimulation with 1μg/mL LPS. The lysates were normalized for total protein using the Biorad protain assay and were subjected to Western blot analysis. Membranes were probed with phospho 536 p65 antibody prior to autoradiography. The curve in the background is IKK activity, provided for a temporal comparison. Data shown are means ± SD of at least 3 replicate experiments. No difference between treatment groups were observed by a one-sided ANOVA with an α of 0.05.

PKCζDN INCREASES PHOSPHORYLATION OF p65 ON SERINE 276

As we have shown that PKCζDN does not block the phosphorylation of p65 through previously described transduction pathways, we decided to determine if serine 276 phosphorylation might represent a novel regulatory mechanism for PKCζ in NF-κB transactivation. Serine 276 phosphorylation is necessary for p65 dependent NF-κB transactivation by mediating the interaction of p300 with p65 [8, 66, 67]. While we are unsure of the signaling events mediating the activation of p65 at serine 276 in NRK cells, PKCζ could modulate phosphorylation of this site through several mechanisms, including Ras, a known activator of MSK1 [116, 117]. In Figure 29, we show that LPS induces the phosphorylation of serine 276 in a time dependent manner and that stable expression of PKCζDN did not reduce this effect, but instead PKCζDN was found to promote a sustained increase in phosphorylation of serine 276 independent of LPS treatment. Furthermore, we show that PKCζ overexpression reduces the maximal phosphorylation of p65 on serine 276, but that phosphorylation of serine 276 is still highly induced by LPS in these cells (Fig 29). We thus conclude that serine 276 is not the site through which PKCζDN blocks transactivation.

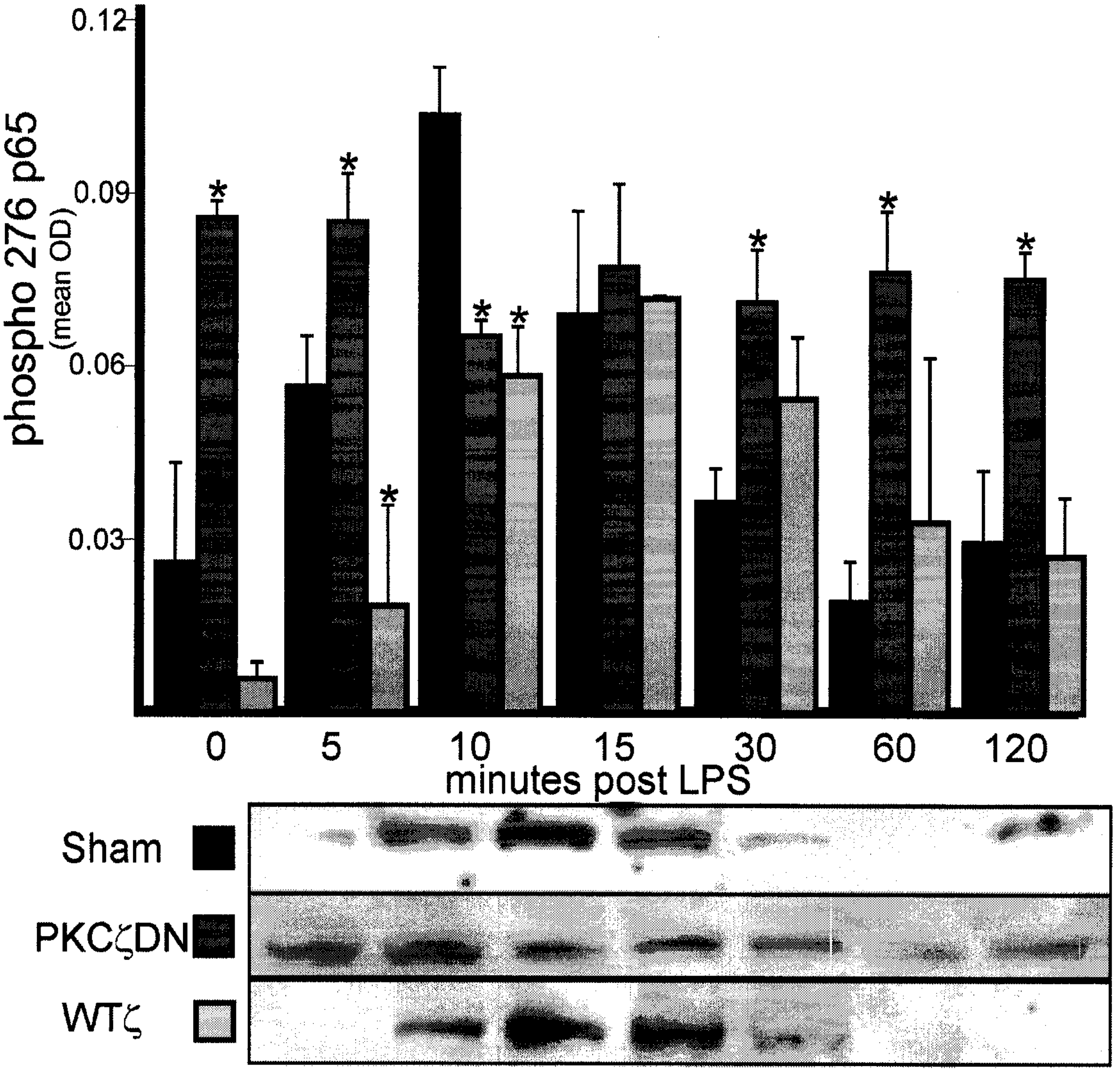

Figure 29. **PKCζDN increases phosphorylation of p65 at serine 276.** Whole cell lysates were taken from cell stably expressing pcDNA3 with an empty target domain (sham), PKCζDN or WTζ after indicated times post LPS tratement. Extracts were standardized for volume and protein concentration using the biorad protein assay and were then subjects to Western blot. Data were generated by autoradiography following incubation with anti p276 p65 and HRP linked secondary. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

PKCζ INHIBITS AKT ACTIVATION, BUT AKT INHIBITON DOES NOT AFFECT TRANSACTIVATION

Having eliminated the most plausible mechanisms through which PKCζ might block phosphorylation events on p65, we turned our attention to PKCζ regulated signals known to alter transactivation of NF-κB independent of p65. Considering that protein kinase B (PKB/AKT) translocation to the membrane and subsequent activation by PDK1 (3 phosphoinositide-dependent protein kinase) is inhibited by PKCζ through the phosphorylation of AKT's phosphoinositide binding pocket, and that AKT is necessary for NF-κB transactivation in some cells, we hypothesized that overexpression of PKCζ inhibits NF-κB transactivation through blockage of AKT [118-121]. We demonstrate that in cells overexpressing either PKCζDN or WTζ, LPS induced phosphorylation of AKT at serine 476 is inhibited (Fig 30). To determine if inhibition of AKT prevents NF-κB transactivation, we measured the ability of the PI3K inhibitors LY6920004 and wortmannin to block NF-κB transactivation. Inconsistent with role for AKT in NF-κB transactivation in this model, we found that PI3K inhibitors do not block NF-κB transactivation (Fig 31). When we checked p65 transactivation directly, we found that AKT inhibition with a pharmacologic AKT inhibitor (AKTi) reduced p65 driven transactivation (Fig 32). Since AKT is critical for the activation of p300 through phosphorylation at Serine 1834, we examined the ability of AKT inhibitors to block p300 transactivation directly [74, 75]. We show that AKT inhibition does not reduce p300 activity (Fig 33). Considering that AKT inhibition reduces p65 transactivation by only 40% and PKCζDN reduces it by 70%, we can not attribute the full inhibition of the PKCζDN to the blockage of AKT activity. Furthermore as AKT inhibition does not block p300 activity, we can not attribute the WTζ blockage of NF-κB transactivation to a reduction in AKT activity. We show that while overexpression of either PKCζDN or WTζ inhibits AKT activation, this inhibition does not fully explain the ability of these vectors to block NF-κB transactivation.

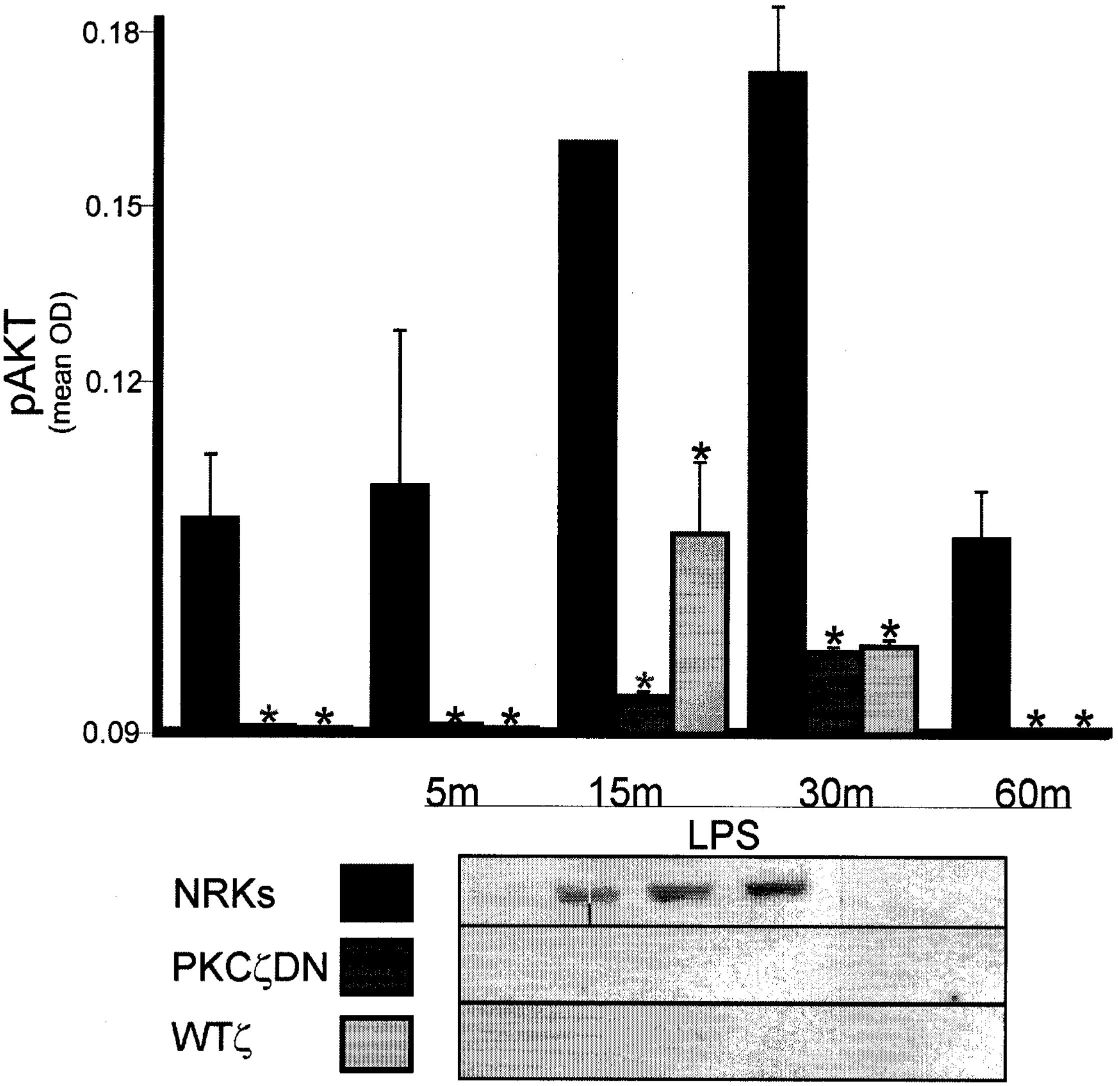

Figure 30. **PKCζ decreases the phosphorylation of AKT.** Whole cells extracts were taken from NRK cells expressing no construct (NRKs), PKCζDN or WTζ at indicated times post treatment. Samples were adjusted to equal volume and equal concentration by Biorad protein assay and then subjected to Western blotting. Membranes were analyzed by probing with anti pp38 followed by autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

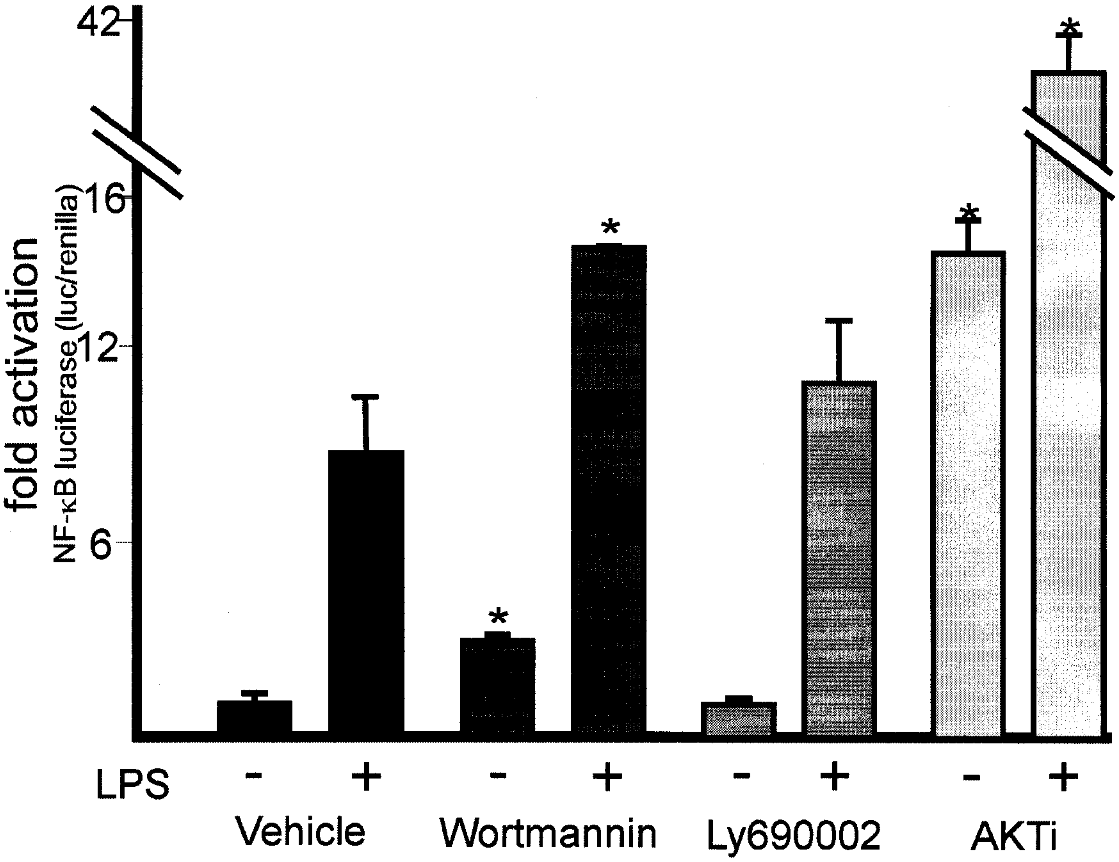

Figure 31. **PI3K/AKT inhibition does not block NF-κB transactivation.** NRK cells were transiently transfected overnight with 4x-luciferase prior to 1h pretreatment with 500nM wortmannin or 3μM LY690002 and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from vehicle (*) were determined by post hoc analysis with Dunnett's post test.

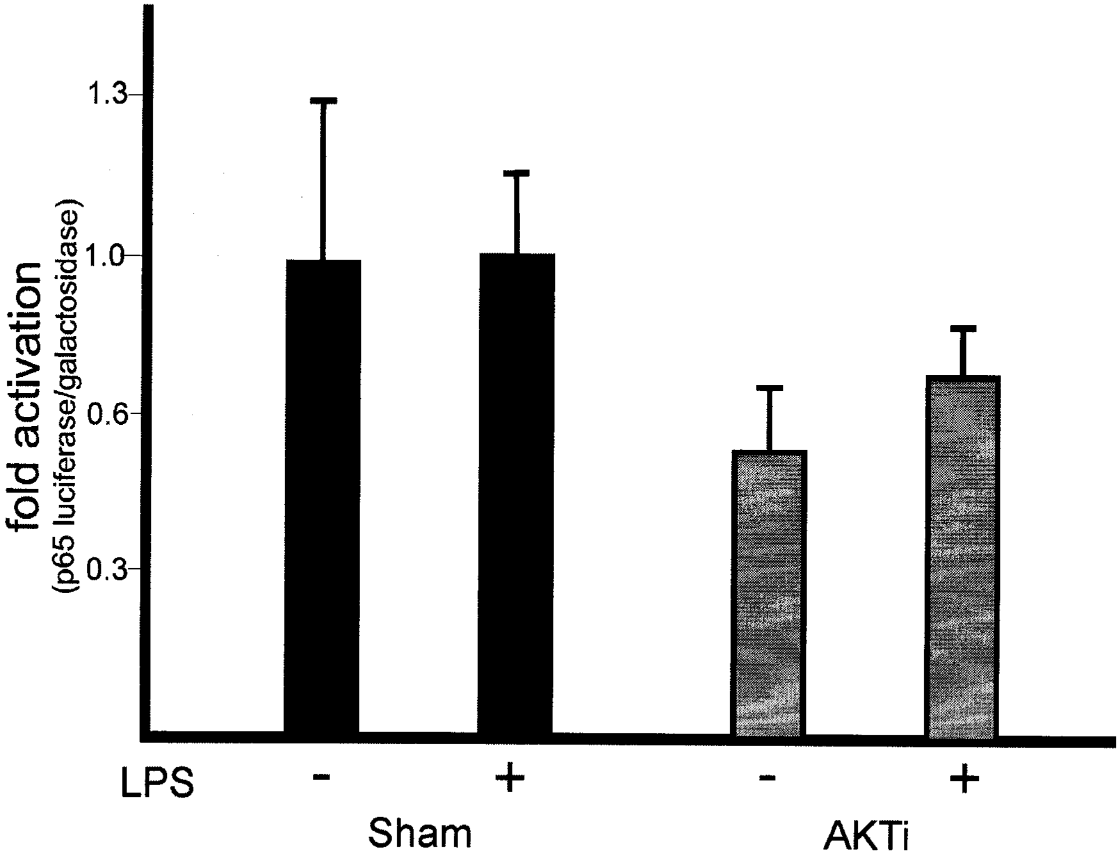

Figure 32. **AKT inhibition does not inhibit p65 luc.** NRK cells were transiently transfected overnight with p65/galactosidase fusion protein and the gal-4 luciferase reporter prior to 1h pretreatment with 10μM AKT inhibitor and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with galactosidase activity. Data shown are means ± SD of at least 3 replicate experiments. No difference was detected by a two-tailed t test with an α of 0.05.

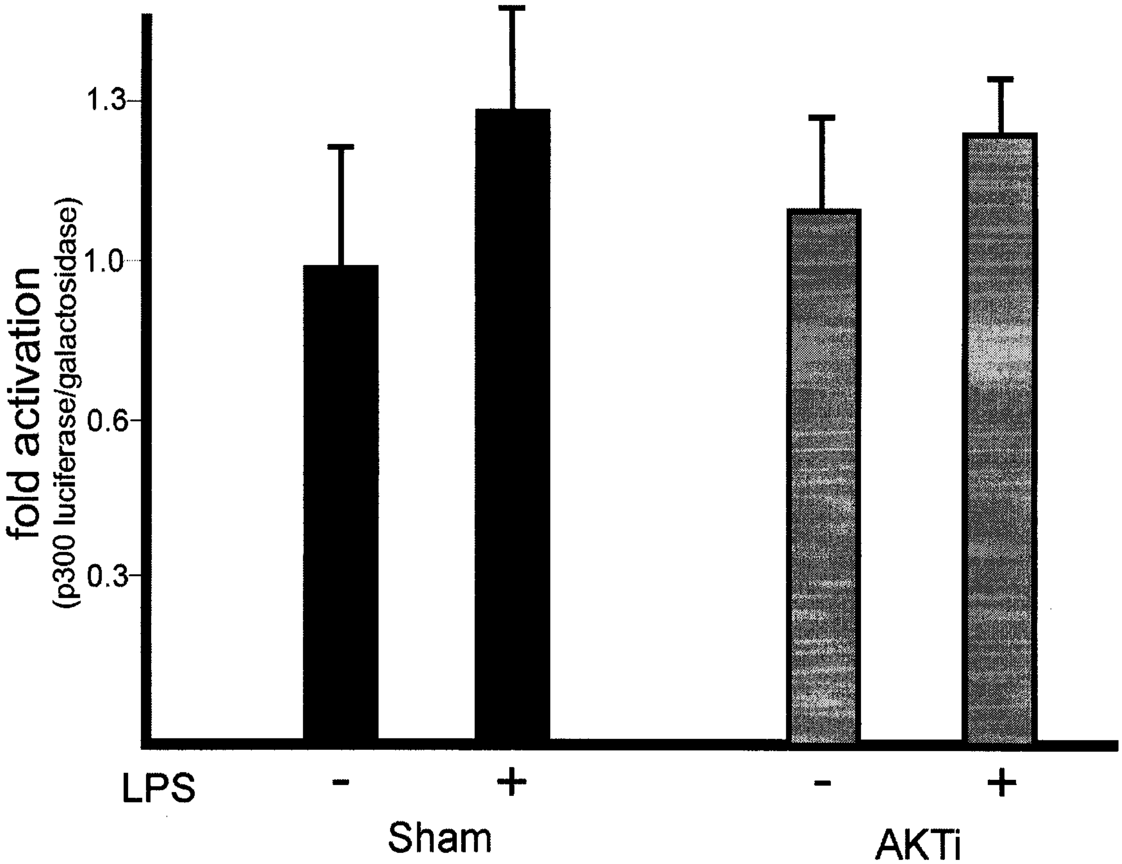

Figure 33. **AKT inhibition does not block p300 luc**. NRK cells were transiently transfected overnight with p300/galactosidase fusion protein and the gal-4 luciferase reporter prior to 1h pretreatment with 10μM AKT inhibitor and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with galactosidase activity. Data shown are means ± SD of at least 3 replicate experiments. . No difference was detected by a two-tailed t test with an α of 0.05.

PKCζDN INHIBITS NF-κB TRANSACTIVATION VIA MEKK1/JNK

As PKCζ inhibition reduces the activation of the MEKK1/JNK axis [122, 123] and JNK is necessary for p65/p300 colocalization [124], we next hypothesized that PKCζDN blocks NF-κB transactivation by preventing JNK mediated p65/p300 colocalization [84]. Consistent with this hypothesis, we show that PKCζDN blocks LPS induced phosphorylation of JNK (Fig 34). Furthermore, we demonstrate that overnight inhibition of JNK results in the blockage of NF-κB transactivation (Fig 35). As MEKK1 activates JNK, we sought to confirm a role for JNK in NF-κB transactivation by measuring NF-κB driven luciferase activity in cells transfected with the constitutively active form of the JNK activator, MEKK1 (pFC-MEKK1) [125]. We show that constitutive transactivation of NF-κB is 5 times higher in pFC-MEKK1 transfected than can be achieved with LPS in normal NRK cells, although LPS may still slightly increase transactivation, although not significantly under these conditions (Fig 36). More importantly, we show that PKCζDN does not block NF-κB transactivation in these cells, but instead increases transactivation, perhaps as a result of the increased phosphorylation of serine 276 as demonstrated in Fig 26 (Fig 36). Surprisingly, we demonstrate that pFC-MEKK1 mediated NF-κB transactivation is not attenuated by expression of WTζ, suggesting that overexpressed PKCζ may also block NF-κB transactivation through a MEKK1 dependent mechanism. To check our findings independent of DNA binding, we measured the ability of pFC-MEKK1 to activate the p65/gal reporter system. Consistent with what we demonstrated using the

4x luciferase, we found that pFC-MEKK1 increases p65 transactivation by 3 fold (Fig 37). These data are consistent with the idea that PKCζDN blocks transactivation of NF-κB by preventing MEKK1/JNK dependent p65/p300 colocalization.

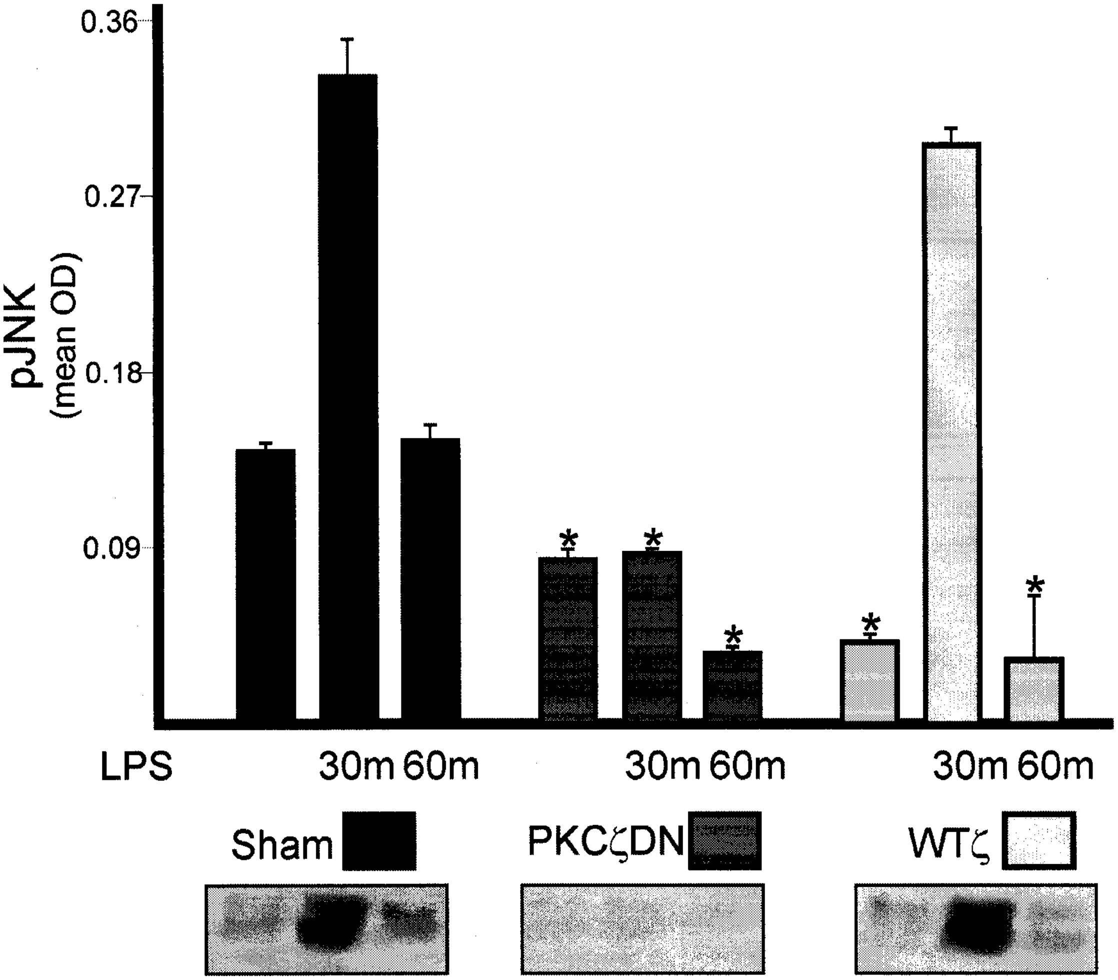

Figure 34. **PKCζDN blocks the phosphorylation of JNK.** Whole cells extracts were taken from NRK cells expressing empty vector (Sham), PKCζDN or WTζ at indicated times post treatment. Samples were adjusted to equal volume and equal concentration by Biorad protein assay and then subjected to Western blotting. Membranes were analyzed by probing with pJNK antibody followed by autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

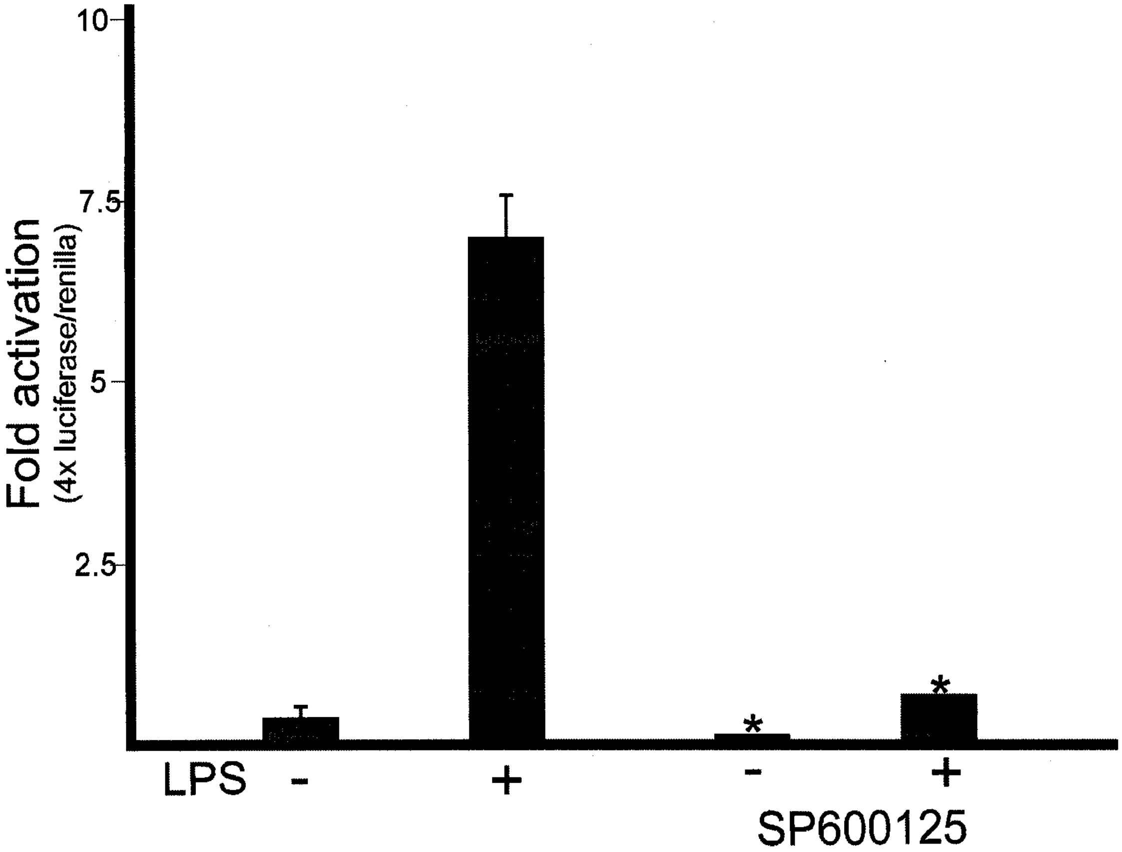

Figure 35. **Long term inhibition of JNK reduces NF-κB transactivation.** NRK cells were transiently transfected overnight with 4x-luciferase prior to 1h or overnight (o/n) pretreatment with 30μM SP600125 and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined using a two-tailed *t*-test. *represents significantly different ($p < 0.05$) from cooresponding sham samples.

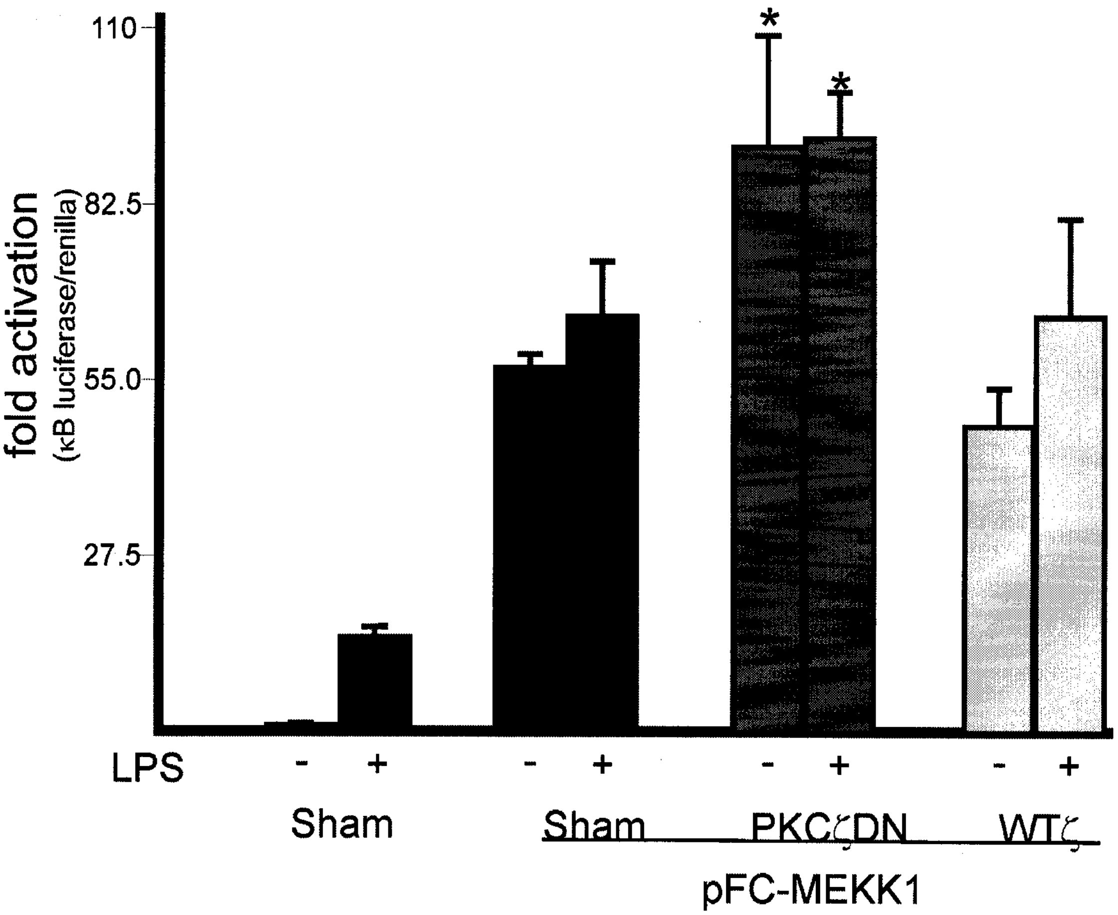

Figure 36. **pFC-MEKK1 activation of NF-κB transactivation is not blocked by PKCζ overexpression.** NRK cells stably expressing empty vector (sham), dominant negative PKCζ (PKCζDN) or wildtype PKCζ (PKCζ) were transfected overnight with 400ng/well 4x-luciferase, 20ng/well CMV driven Renilla luciferase and 200μg/mm^2 pFC-MEKK1. The cells were harvested in passive lysis and analyzed by luminescence according to Promega's protocol after 4h stimulation with 1μg/mL LPS. These data are represented as fold activation of the sham transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. The first two lanes are historical control and are not transfected with pFC-MEKK1. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA using an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

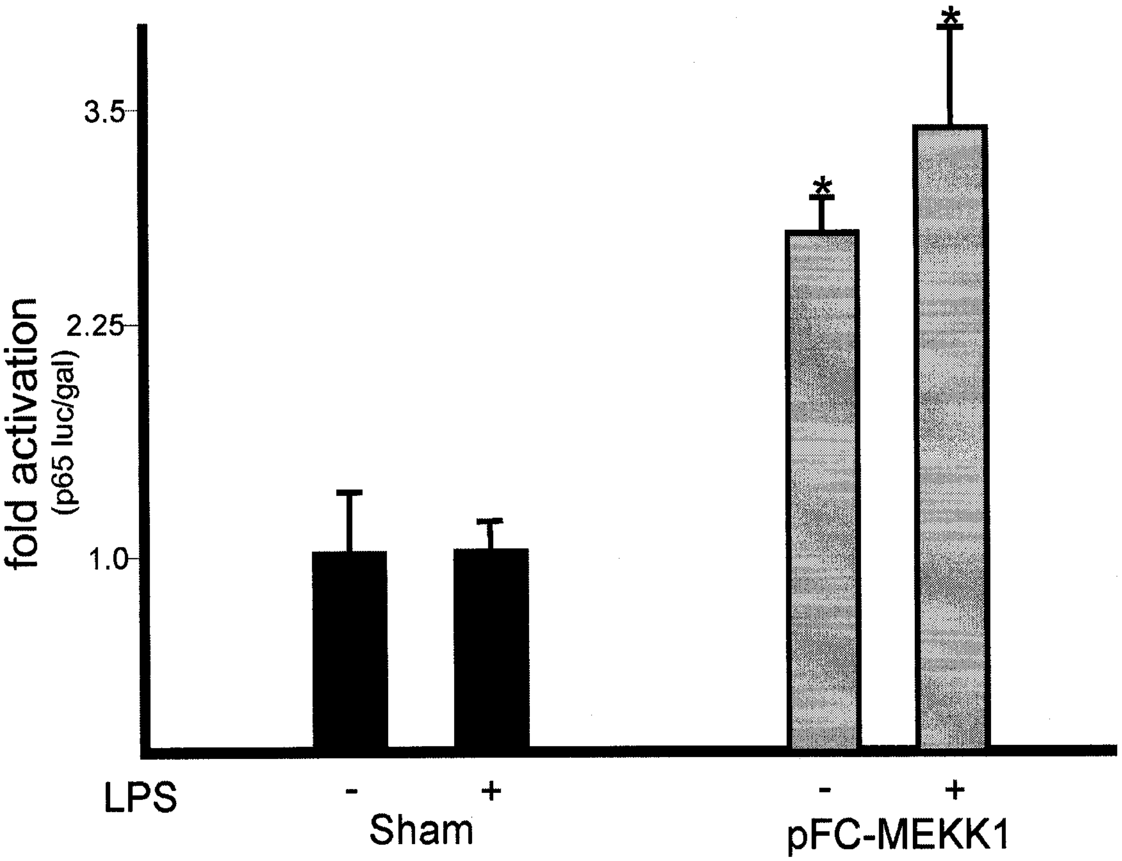

Figure 37. **pFC-MEKK1 increases p65 luciferase**. NRK cells were transiently transfected overnight with pFC-MEKK1, p65/galactosidase fusion protein and the gal-4 luciferase reporter prior to 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with galactosidase activity. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined using a two-tailed *t*-test. *represents significantly different ($p < 0.05$) from cooresponding sham samples.

PKCζ OVEREXPRESSION BLOCKS NF-κB TRANSACTIVATION THROUGH p38

In Figure 31, we demonstrate that PKCζ overexpression does not inhibit basal or LPS inducible NF-κB transactivation when pFC-MEKK1 is coexpressed, suggesting that pFC-MEKK1 can recover transactivation. Having already demonstrated that PKCζ overexpression does not prevent the phosphorylation of JNK (Fig 34), we hypothesized that PKCζ overexpression blocks NF-κB transactivation by preventing the activation of p38, the other stress kinase activated by pFC-MEKK1 known to regulate NF-κB transactivation [126-130]. In NRK cells, we found that overexpression of PKCζ blocks the phosphorylation of p38 (Fig 38). Furthermore, we find that inhibition of p38 prevents NF-κB transactivation (Fig 39). Since p38 has been previously demonstrated to impact NF-κB signaling through modulation of p300 (135), we measured p300 activity in the presence of the p38 inhibitor SB203580 and found that blockage of p38 reduces p300 activity (Fig 40). We confirm that inhibition of NF-κB transactivation by the overexpression of PKCζ is associated with the blockage of p38 activation of p300 activity.

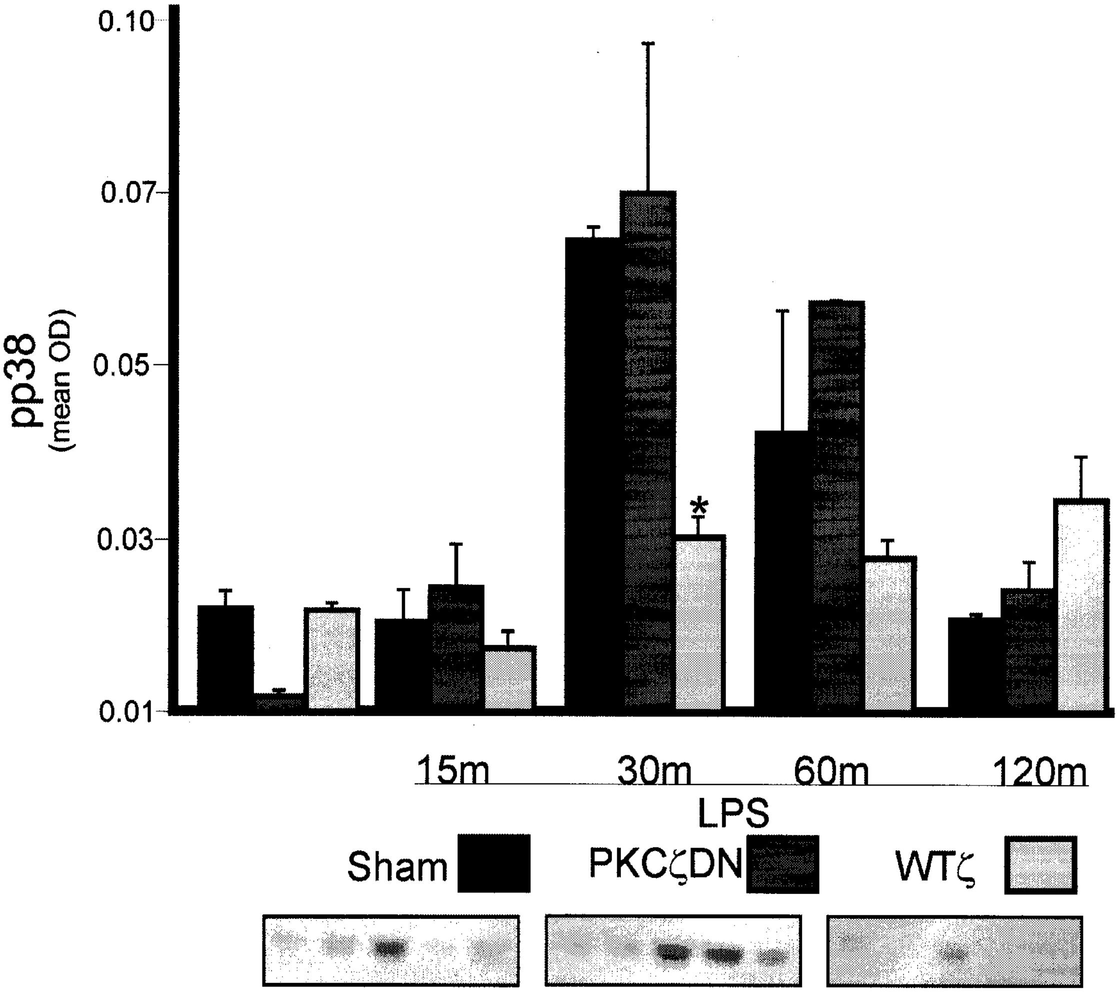

Figure 38. **PKCζ blocks the phosphorylation of p38.** Whole cells extracts were taken from NRK cells expressing empty vector (sham), PKCζDN or WTζ at the indicated times post treatment. Samples were adjusted to equal volume and equal concentration by Biorad protein assay and then subjected to Western blotting. Membranes were analyzed by probing with pp38 antibody followed by autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

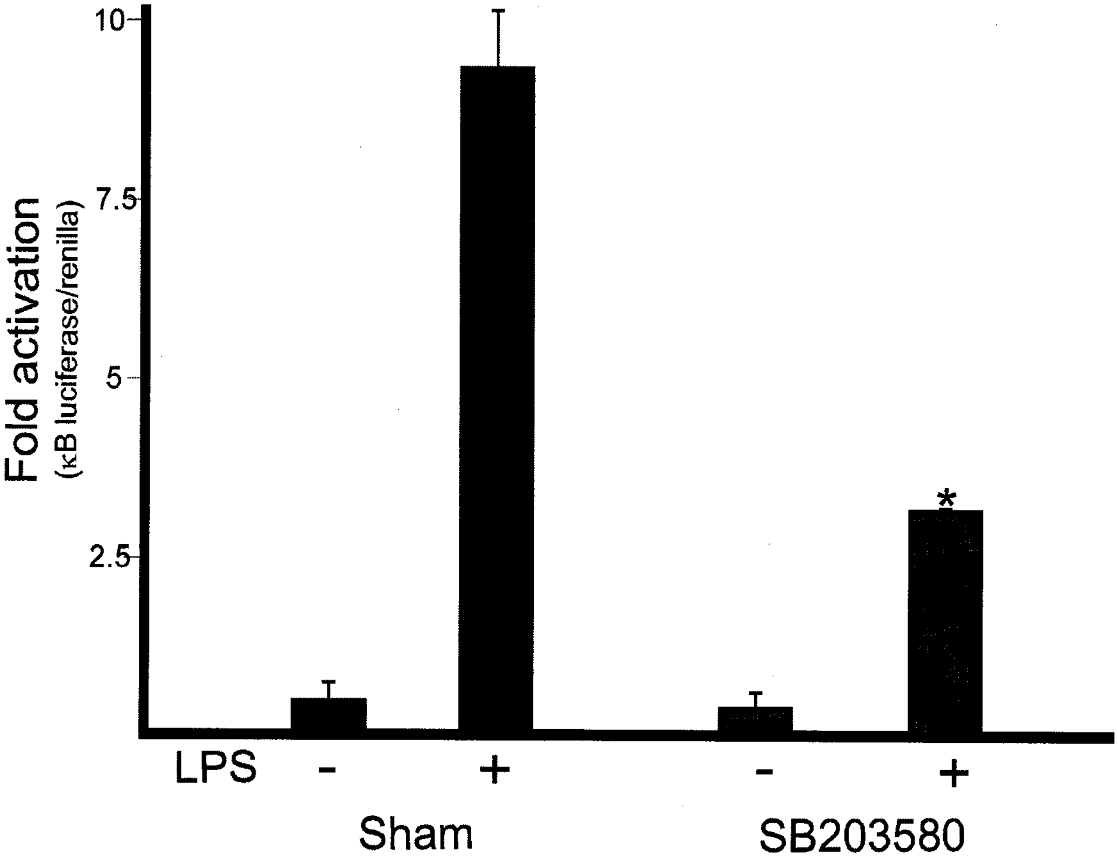

Figure 39. **SB203580 blocks NF-κB transactivation.** NRK cells were transiently transfected overnight with 4x-luciferase prior to 1h or overnight (o/n) pretreatment with 30μM SB203580 and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis buffer and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with the Renilla luciferase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined using a two-tailed *t*-test. *represents significantly different ($p < 0.05$) from cooresponding control samples.

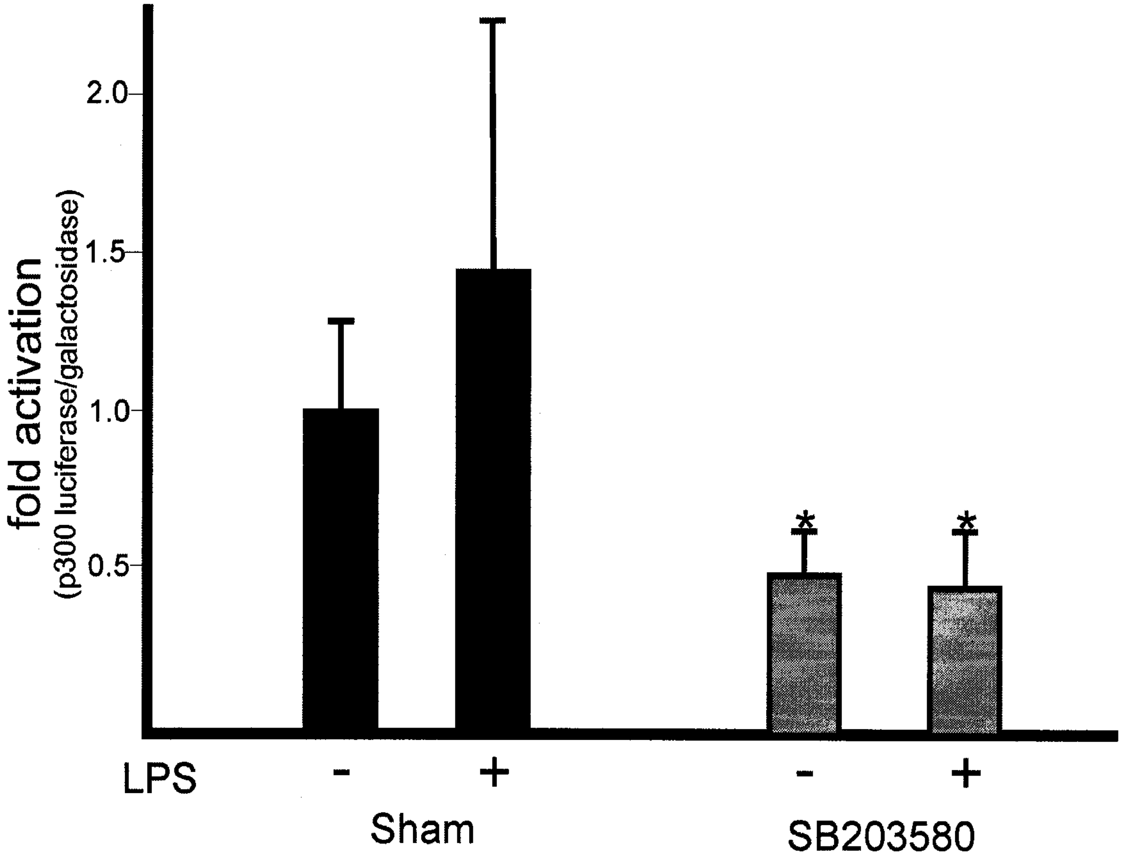

Figure 40. **SB203580 blocks activation of the p300 reporter system.** NRK cells were transiently transfected overnight with p300/galactosidase fusion protein and gal4 luciferase reporter prior to 1h or overnight (o/n) pretreatment with 30μM SP600125 and 4h stimulation with 1μg/mL LPS. The cells were harvested in passive lysis and analyzed by luminescence according to Promega's protocol. These data are represented as fold activation of the vehicle transfected constitutive level of luciferase after correction for transfection efficiency with the galactosidase. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined using a two-tailed *t*-test. *represents significantly different ($p < 0.05$) from corresponding sham samples.

PKCζ OVEREXPRESSION CHANGES EXPRESSION OF GENES IMPORTANT TO IMMUNE RESPONSE

NF-κB translocation, DNA binding and transactivation culminates in the expression of a wide variety of genes, especially those involved in the inflammatory response [131]. After demonstrating that overexpression of wildtype and catalytically inactive forms of PKCζ result in changes in NF-κB translocation and transactivation, we hypothesized that stable expression of PKCζ, irrespective of catalytic activity, decreases NF-κB mediated gene expression. Tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) have been extensively characterized as NF-κB mediated genes, [128, 132, 133] and thus we measured these in NRK cells stably expressing PKCζDN or WTζ. We quantified cDNA generated from the mRNA of NRK cells treated with 1μg/mL LPS for 4h and show that either PKCζDN or WTζ prevents the increase in TNFα mRNA after LPS treatment, consistent with our finding in Figure 21 that either PKCζDN or WTζ overexpression inhibit NF-κB transactivation (Fig 41). However, we show that IL-6 mRNA is increased by either PKCζDN or WTζ expression and trends upward in the PKCζDN line, albeit with high variance, as compared to the sham transfected cells (Fig 42). TNFα and IL-6 are often induced together after stimulation of the inflammatory response, but during inflammatory resolution, the switch from innate to acquired immunity requires high IL-6 expression without TNFα expression [134, 135]. Consistent with our assertion that expression of either PKCζDN or WTζ induces an inflammatory resolution, we show that either PKCζDN or WTζ increases A20, a feedback inhibitor of IKK associated with the termination of NF-κB signaling (Fig 43) [136-138]. We demonstrate that under conditions known to prevent NF-κB transactivation, the expression of either PKCζDN or WTζ reduces the expression of a critical gene responsible for the activation of innate immunity, TNFα and increases the expression of genes responsible for the termination of innate immunity, IL-6 and A20.

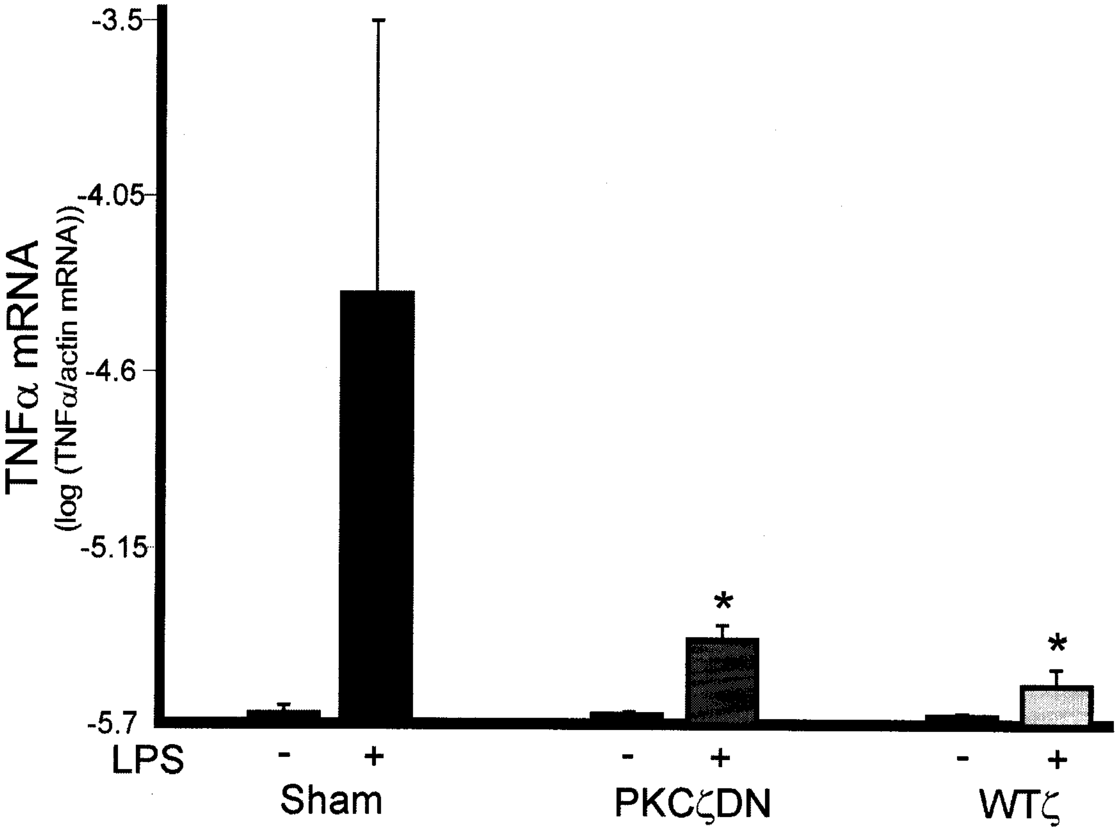

Figure 41. **PKCζ overexpression decreases LPS induced TNFα mRNA.** NRK cells stably expressing empty vector (sham), dominant negative PKCζ (PKCζDN) or wildtype PKCζ (PKCζ) were harvested by lysis in Trizol® after 4h stimulation with 1μg/mL LPS. RNA was extracted according manufacturers protocols and was converted to cDNA for analysis on an ABI 7900HT Fast Real-Time PCR System according to materials and methods. Data are displayed as log transformed ratio of TNFα mRNA to β-actin mRNA. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

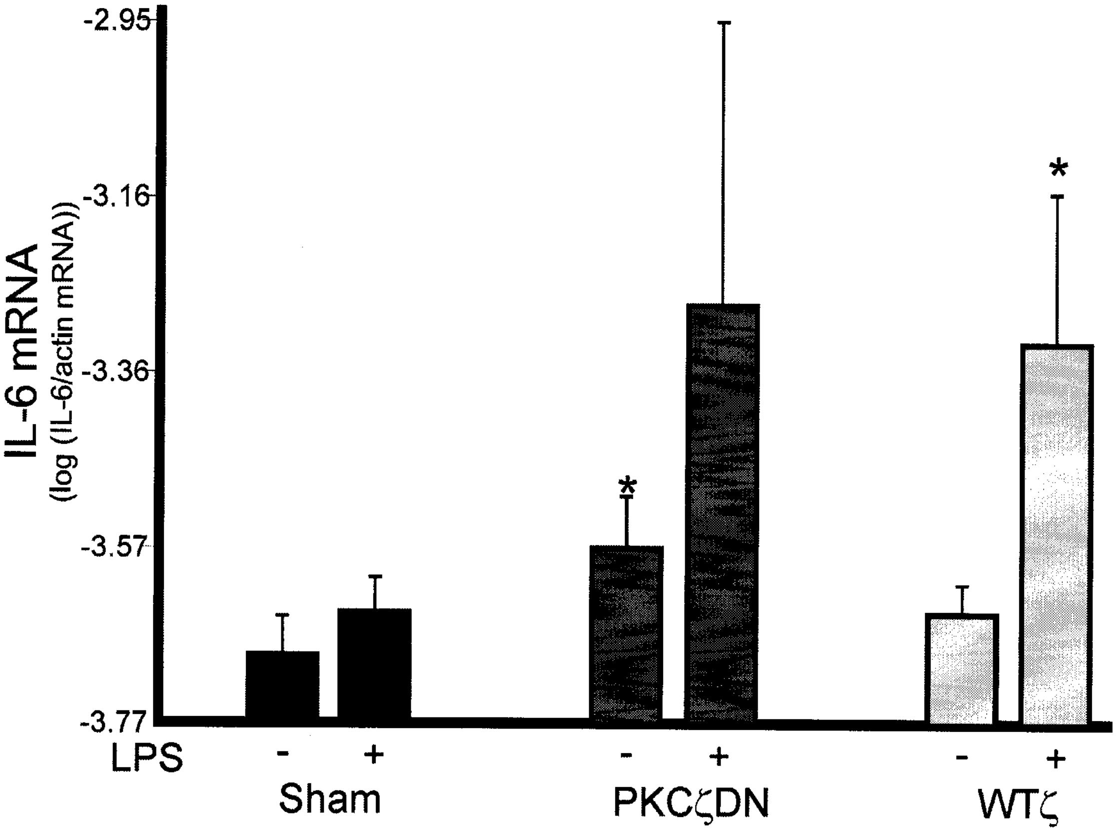

Figure 42. **PKCζ overexpression increases LPS-induced IL-6 mRNA.** NRK cells stably expressing empty vector (sham), dominant negative PKCζ (PKCζDN) or wildtype PKCζ (WTζ) were harvested by lysis in Trizol® after 4h stimulation with 1μg/mL LPS. RNA was extracted according manufacturers protocols and was converted to cDNA for analysis on an ABI 7900HT Fast Real-Time PCR System according to materials and methods. Data are displayed as log transformed ratio of IL-6 mRNA to β-actin mRNA. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

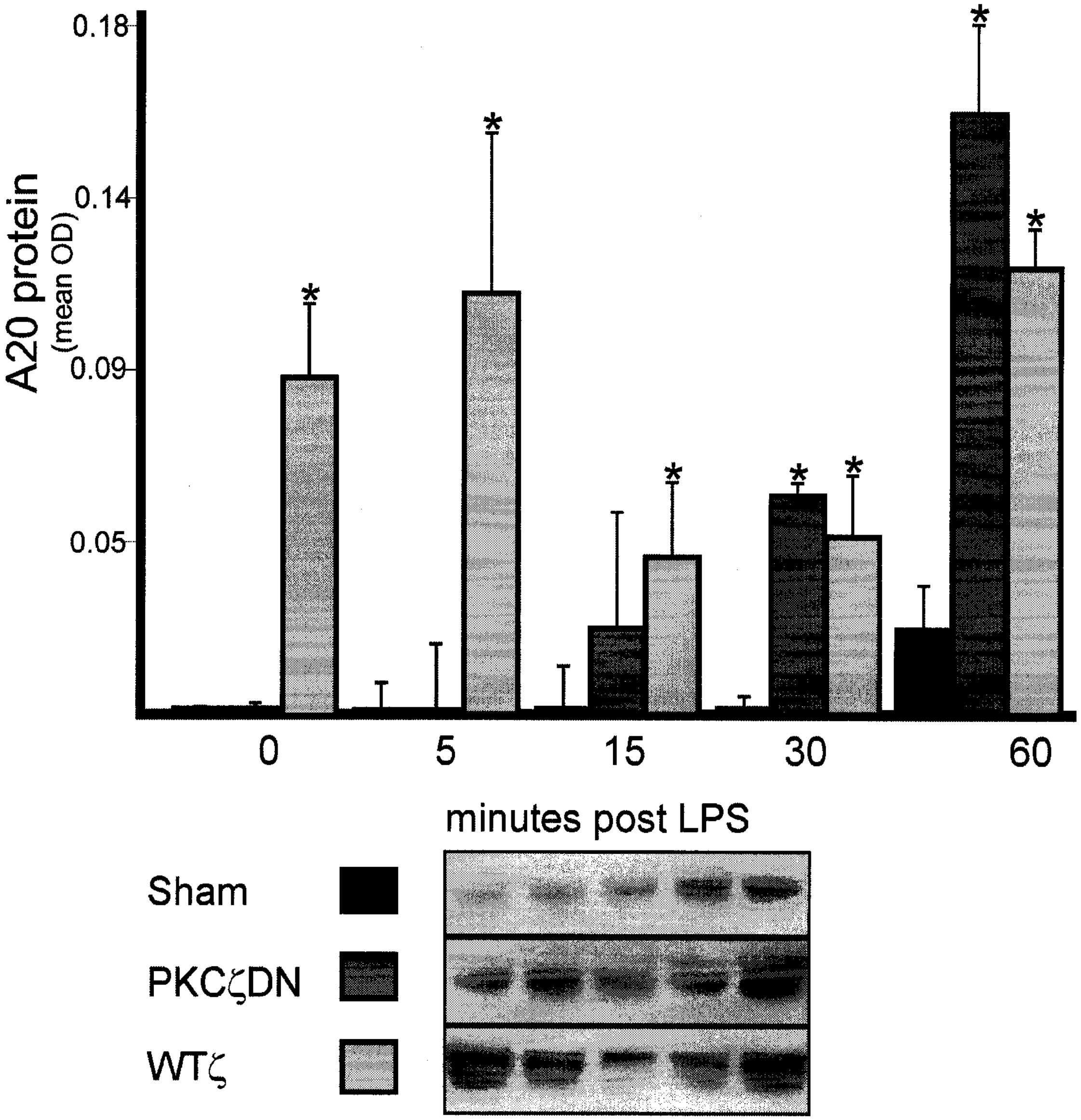

Figure 43. **PKCζ overexpression increases LPS-induced A20 protein.** NRK cells stably expressing empty vector (sham), dominant negative PKCζ (PKCζDN), or wildtype PKCζ (WTζ) were stimulated with 1μg/mL LPS for indicated times before collection of whole cell lysates. Protein was equalized using the Biorad protein assay to ensure equal protein loading in Western blot. Membranes were probed with A20 antibody prior to autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

PKCζ BLOCKS NF-κB-INDEPENDENT INFLAMMATORY GENE INDUCTION

As the changes in NF-κB mediated gene expression down stream of either PKCζDN or WTζ overexpression are consistent with the resolution of the innate immune response, we sought to determine if PKCζ overexpression negatively regulates the NF-κB independent inflammatory Cox-2. We demonstrate that WTζ expression decreases, whereas PKCζDN increases Cox-2 expression, which is independent of NF-κB in these cells (Fig 44) [139]. Since p38 can regulate Cox-2 expression, we attribute the inhibition of Cox-2 by PKCζ overexpression on the decrease in p38 activation in these cells (Fig 38) [127]. As Cox-2 generates prostaglandins responsible for the recruitment of the innate immune response and can facilitate early inflammation, our finding is consistent with the idea that PKCζ acts through NF-κB-dependent and independent mechanisms to regulate the switch from innate to acquired immunity in these cells [140, 141].

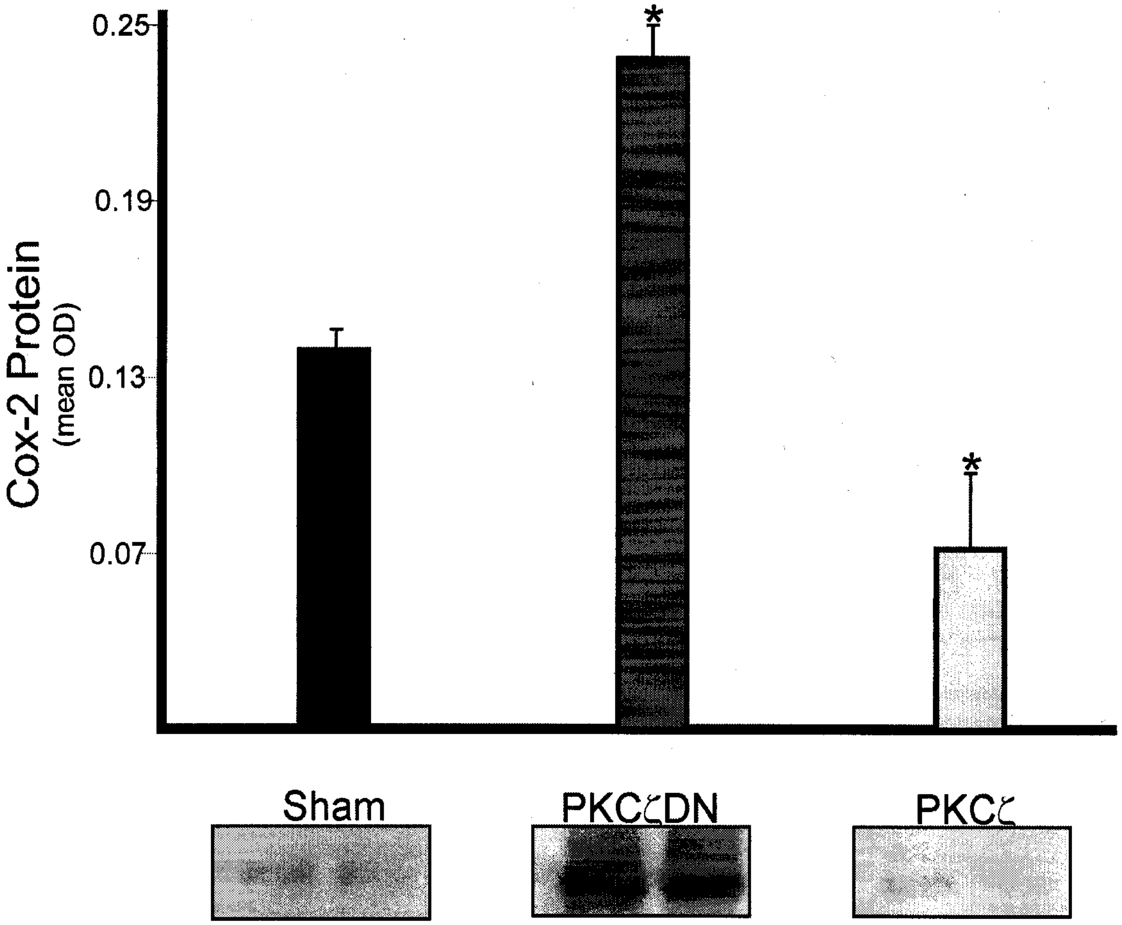

Figure 44. **Constitutive Cox-2 expression is negatively regulated by PKCζ.** Whole cell lysates were taken from NRK cells stably overexpressing empty vector, dominant negative PKCζ (PKCζDN), or wildtype PKCζ (PKCζ) with 1μg/mL LPS. We found that stable expression of PKCζDN increased and WTζ decreased Cox-2 expression in controls at all times tested (5m, 10m, 15m, 30m, 60m and 120m post LPS treatment), so for simplicity only the nonstimulated NRK cells are displayed here. The lysates were normalized for total protein using the biorad protein assay and were subjected to Western blot analysis. Membranes were probed with anti Cox-2 prior to autoradiography. Data shown are means ± SD of at least 3 replicate experiments. Differences between treatment groups were determined with a one-way ANOVA with an α value of 0.05. Data points significantly different from sham (*) were determined by post hoc analysis with Dunnett's post test.

CHAPTER 5: DISCUSSION

PKCζ is a critical signaling intermediate in the NF-κB cascade, responsible for the accumulation of NF-κB-DNA binding via IKK and for transactivation directly through phosphorylation of the p65 subunit on serine 311 [72, 107]. This study is the first to our knowledge to demonstrate that PKCζ regulates NF-κB transactivation through modulation of p300 activity. We define in this work a unified role for PKCζ in regulating NF-κB where PKCζ acts to promote DNA binding and transactivation by stimulating IKK activation, p65/p300 colocalization and p300 activity in a manner consistent with the resolution of the innate inflammatory response after LPS treatment.

PKCζ is a well established activator of NF-κB DNA binding and transactivation, however the recent study by Banan et al [105] suggests that PKCζ may also inhibit NF-κB activity to preserve the integrity of the epithelial barrier. We expand on these findings by showing that PKCζ acts as both an inhibitor and an activator of LPS induced TNFα expression in NRK cells (Figure 45). We show that PKCζ is able to regulate NF-κB in both a positive and negative manner by segregating those regulatory actions, as transient activation of PKCζ induces IKK activation and p65/p300 colocalization, whereas long term overexpression of active PKCζ inhibits p300 activity. The precise signaling cascades through which PKCζ inhibits NF-κB transactivation in this model are unknown, however our demonstration that prevention of p65/p300 binding is associated with JNK activity in PKCζDN cells is consistent with the previous finding that JNK directly mediates this interaction [124]. Further investigation will be required to determine the precise action of p38 in the activation of p300, as p38 phosphorylation of p300 is often associated with the degradation of p300 [142]. One intriguing possibility arises from the finding that p38 can inhibit PKCζ activity [143], so p38 could activate p300 by a feedback loop that blocks PKCζ mediated inhibition of HAT activity.

This study further defines the role of PKCζ in the activation of NF-κB in renal proximal tubular cells. The current observations are consistent with previous experiments by Moscat et al describing a need for PKCζ in mediating IKK activity in wildtype cells and in mediating NF-κB transactivation [112]. The binding of PKCζ to the signalasome implies a direct effect in the activation of IKK, but it is clear that an extra signal is required for the activation of IKK, as PKCζ overexpression is not sufficient for the activation of IKK. Furthermore, the findings that overexpression of either PKCζDN or PKCζ results in a decrease in NF-κB transactivation through separate mechanisms, demonstrates a need for an additional control mechanism(s) to activate PKCζ, while also limiting the inhibitory action of PKCζ on transactivation in a rheostat-like phenomenon. Notably, MEKK1 represents a single PKCζ regulated kinase that displays all the necessary criteria to act as a single intermediate between PKCζ and both IKK activation and NF-κB transactivation [122]. Multiple forms of MEKK1 make such a complex regulatory mechanism possible. MEKK1 exists as a 196kDa protein with one of the longest regulatory domains reported for a protein kinase. Full length MEKK1 is activated by removal of the inhibitory domain from the catalytic domain by either post-translational modifications that prevent the interaction of the two domains or by cleavage between the two domains, liberating the catalytic domains. Upon activation, MEKK1 phosphorylates IKK [144-146] and the upstream components of the JNK cascade [147]. As PKCζDN cells have impaired IKK and JNK activation, we believe that PKCζDN blocks binding and transactivation by blocking MEKK1 activation [147]. We demonstrate that consistent with this idea, active MEKK1 recovers NF-κB transactivation in cells expressing of PKCζDN. Overexpression of pFC-MEKK1 can activate p38 in addition to JNK and IKK, possibly explaining the supraphysiologic activation of NF-κB transactivation demonstrated here with this plasmid. However, no direct catalytic activity is known which links MEKK1 kinase with the p38 pathway in mammals [144]. We propose here that overexpression of PKCζ blocks the crosstalk mechanism through which MEKK1 activates p38, thus preventing p38 activation and NF-κB transactivation in a MEKK1 dependent manner.

LPS induced NF-κB DNA binding and transactivation culminates in the expression of multiple genes, many of which responsible for activation of the innate inflammatory response [34, 148, 149]. We show here that PKCζ acts to reduce inflammation by preventing the expression of TNFα, a strong chemoattractant for monocytes and increasing the expression of IL-6, an inhibitor of PMN attachment to epithelial cells, and A20, the intracellular feedback inhibitor of NF-κB (Figure 46) [50, 134, 137]. IL-6 is often viewed as an inflammatory stimulus, but it represents a critical 'switch' in the inflammatory resolution process, as it inhibits innate immunity and initiates acquired immunity [134]. Furthermore, we demonstrate that PKCζ inhibition of the inflammatory response comprises more than the NF-κB pathway, as Cox-2 has been demonstrated in these cells to be independent of NF-κB activation [139]. As a wide number of inflammatory genes, including Cox-2, require p300 activity for expression, we are in the process of determining the full scope of PKCζ mediated inflammatory inhibition in these cells.

In conclusion, we show that PKCζ is a critical determinant in the response of NRKs to LPS stimulation. We demonstrate both positive and negative roles for PKCζ in the regulation of NF-κB transactivation and subsequent expression of genes involved in the innate immune response. Our work suggests that PKCζ and MEKK1 may represent potential avenues for therapeutic intervention in cases of septicemia, acute renal failure and transplant rejection, as inflammatory induced renal failure is a critical determinant for survival in these conditions.

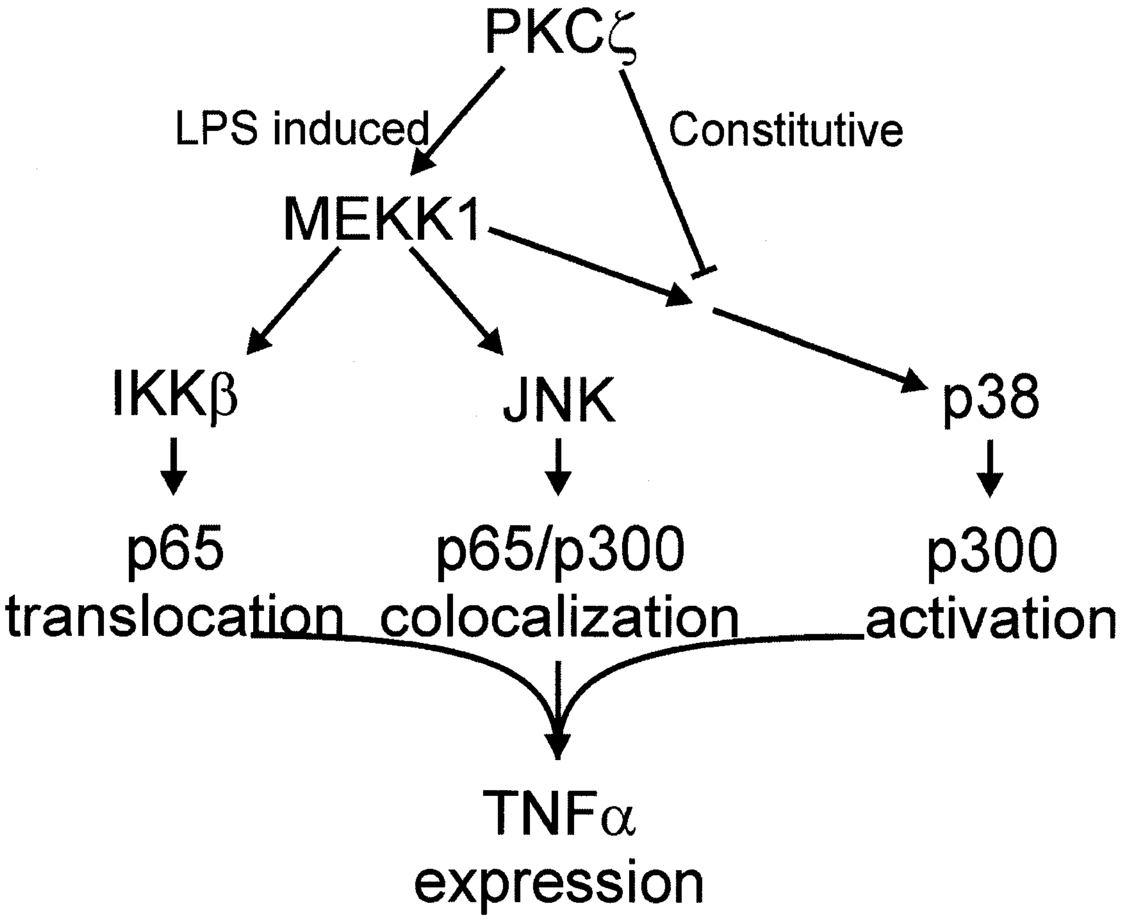

Figure 45. **PKCζ regulates NF-κB gene expression through multiple mechanisms.** LPS induces p65 translocation and p65/p300 colocalization through PKCζ mediated activation of MEKK1, which results in the activation of IKKβ and JNK, respectively. Long term constitutive activation of PKCζ prevents TNFα expression by blocking p300 activation in these kidney cells. We speculate that MEKK1 can regulate p38 activation through crosstalk with an unknown pathway.

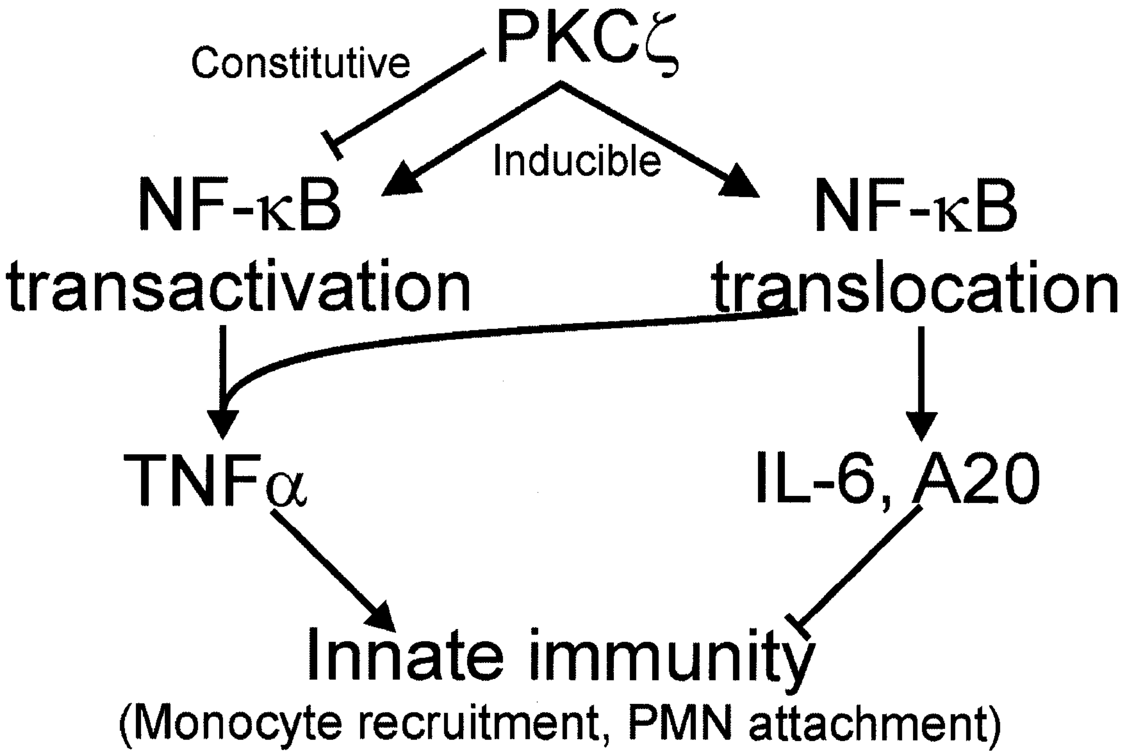

Figure 46. **PKCζ regulates innate immunity through both positive and negative mechanisms.** Inducible activation of PKCζ increases NF-κB translocation to the nucleus and transactivation, resulting in the inducible expression of TNFα and subsequent activation of the innate immune response. Whereas, constitutively high activation of PKCζ results in the inhibition of NF-κB transactivation and switches the immune reponse away from innate immunity by increasing the expression of the NF-κB inhibitors IL-6 and A20.

CHAPTER 6: CONCLUSIONS

1. Inhibition of PKCζ with a dominant negative PKCζ or pseudosubstrate blocks LPS induced translocation of NF-κB.
2. PKCζ inhibitors block LPS induction of IKK activity, as measured by an *in vitro* activity assay and by the phosphorylation of the endogenous target of IKK, IκBα.
3. PKCζ interacts with IKK after LPS stimulation prior to the activation of IKK.
4. Stable expression of PKCζDN slightly reduces maximal IKK activation, but increases NF-κB DNA-binding.
5. PKCζ inhibitors reduce NF-κB transactivation by blocking the activation of the p65 subunit and the subsequent colocalization of p300.
6. PKCζ overexpression blocks NF-κB transactivation by reducing p300's transactivating potantial.
7. Phosphorylation of p65 on serine 311 is not blocked by overexpression of either PKCζDN or PKCζ, and thus is not the mechanism through which either block NF-κB transactivation.
8. Phosphorylation of p65 on serine 536 is not blocked by overexpression of either PKCζDN or PKCζ, and this is not the mechanism through which either block NF-κB transactivation.

9. Phosphorylation of p65 on serine 276 is enhanced by the overexpression of PKCζDN and is not affected by the overexpression of active PKCζ and thus is not the mechanism through which either block NF-κB transactivation.
10. PKCζDN inhibition of AKT may contribute to the inhibition of NF-κB transactivation by PKCζDN, but AKT inhibition is less potent than PKCζDN at blocking activation and thus does not fully explain the action of PKCζDN.
11. PKCζ inhibition of AKT activity is not associated with the ability of overexpressed PKCζ to block NF-κB transactivation, as AKT inhibition does not block the activity of p300.
12. PKCζDN inhibition of JNK is associated with blockage of NF-κB transactivation, and defines a novel mechanism through which PKCζ activates NF-κB transactivation.
13. PKCζ inhibition of p38 is associated with blockage of NF-kB transactivation, and defines a novel mechanism through which PKCζ inhibits NF-κB transactivation.
14. Blockage of NF-κB transactivation by overexpression of either PKCζDN or PKCζ, reduces LPS induced expression of the proinflammatory mediators TNFα and Cox-2.
15. Blockage of NF-κB transactivation by overexpression of either PKCζDN or PKCζ increases the LPS induced expression of the inflammatory resolution mediators IL-6 and A20.

16. The change in the gene profile resulting from the blockage of NF-κB transactivation by overexpression of either PKCζDN or PKCζ is indicative of a model of inflammatory resolution and may represent a critical determinant in kidney function in cases of sepsis.

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