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EXPERIMENTAL STUDY

Regulation of cerebrovascular endothelial peroxisome proliferator activator receptor alpha expression and nitric oxide production by clofibrate

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Abstract: *Background:* Peroxisome proliferator activator receptor alpha (PPARα), a member of the nuclear receptor superfamily, is known to increase nitric oxide (NO) production and the mechanisms by which PPARα activation alleviates vascular dysfunction may predicate its activation and possible expression.
Objectives: We have evaluated the effects of acute clofibrate, a PPARα ligand and the role of PKC on PPARα expression and NO production in cultured cerebral microvascular endothelial cell (CMVEC).
Methods: Confluent CMVEC derived from pig brain were cultured and the role of PKC in acute clofibrate-induced PPARα expression and NO production was determined in the presence or absence of PKC activator phorbol myristate acetate (PMA) or inhibitor (calphostin C).
Results: Incubation of CMVEC with clofibrate or PMA increased NO production by 40 % or 27 %, respectively, whereas co-incubation of cells with PMA and clofibrate had no effect on NO production. Incubation of cells with Calphostin C blunted PMA but not clofibrate-induced increase in NO production. L-NAME (0.1 mM), an inhibitor of NO synthase, reduced basal (47 %; $p<0.01$) and abolished clofibrate-induced increase in NO production. Clofibrate increased PPARα expression (26 %; $p<0.05$) while PMA with or without clofibrate reduced PPARα expression ($p<0.01$). On the other hand, calphostin C reduced basal (69 %, $ap<0.01$) as well as clofibrate-induced increase (59 %, $p<0.01$) in PPAR expression, and further reduced PMA-induced down regulation of PPAR expression. eNOS expression was not significantly affected by either clofibrate or PMA, alone or in combination.
Conclusion: These results show that in the brain microvascular endothelial cell, PPARα activation increases NO production-independent of eNOS and PKC signaling pathways, a regulates PPARα expression through a complex PKC signaling mechanism(s) as both PKC activation and inhibition reduced clofibrate-induced activation of PPAR expression *(Fig. 4, Ref. 32)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: clofibrate, peroxisome proliferator activator receptor alpha, protein kinase C, cerebral microvessel, endothelial cells.

Abbreviations: protein kinase C (PKC), peroxisome proliferator activator receptor (PPAR), phorbol-myristate acetate (PMA), cerebral microvascular endothelial cell (CMVEC).

Cerebral microvascular endothelial cells (CMVEC) play an important role in the modulation of cerebrovascular tone maintaining vascular patency through production of endothelial cell-derived dilator agents such as nitric oxide (NO), hyperpolarizing factor (EDHF) and/or constrictor agents e.g. endothelin-1 (ET-1) (1, 2, 3). Interactions between the vascular dilator and contractile mediators produced by these agents maintain normal vascular tone and derangement in the production of one or more of these agents leads to endothelial dysfunction with serious health consequences. Several disease conditions especially cardiovascular diseases have been linked to an imbalance in the production/actions of dilator and contractile products of the vascular system and have been the basis of therapeutic target. Therefore, understanding the vascular regulatory processes is very important in identifying potential therapeutic agents. Hypolipidemic agents that are ligands for PPAR e.g. clofibrate, have been shown to improve vascular function and the mechanism(s) by which this occurs is still not completely understood. However, it has been suggested athat PPAR activation by hypolipidemic agents and their effect on endothelial nitric oxide synthase (eNOS)-derived NO production accounts for their protective effect in vascular diseases (4, 5, 6).

NO is produced by the conversion of L-arginine to L-citrulline by the action of NOS. There are 3 different isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible

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NOS (iNOS). eNOS is considered the most important as it is essential for the maintenance of cardiovascular integrity by producing NO, a potent vasodilator that possesses anti-proliferative effect on vascular smooth muscle cells, as well as anti-thrombotic and anti-platelet aggregatory effects (1, 7). Therefore, regulation of the nitric oxide systems is very important for the regulation of vascular function. PKC has been reported to be one of the potent regulators of NO production in the vascular system (1, 8, 9, 10). Increased PKC can occur following activation of membrane receptor resulting in the stimulation of phospholipase C and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) producing inositol 1,4,5-triphosphate (IP3), increased Ca^{2+} and the subsequent activation of PKC, translocation, and increased expression (11, 12). PKC is known to regulate various vascular functions such as release of vasoactive agents by vascular cells and vascular reactivities and has been shown to play a role in the pathology of vascular diseases (10, 13, 14, 15). Hence, preservation of the normal function of NO can be a therapeutic target for the amelioration of vascular dysfunction such as suggested for the beneficial effects of peroxisome proliferators activated receptor (PPAR) activation in the vascular system (16–18).

Recently, alleviation of vascular dysfunction by PPAR ligands has been reported (16–19) but the mechanisms involved are still not clear. However, evidence has been presented suggesting that the vascular actions of PPARα activation may involve increased endothelial cell-derived NO production and possibly mediated through PKC signaling pathways (15, 18). In addition, a mechanism involving PKC has been reported to account for PPAR activation in human keratinocyte and hepatocytes (20, 21). However, in the brain, the mechanism behind PPARα-induced increased NO production is still not clear; and the role of PKC in acute PPARα activation and modulation of NO system in cerebral microvascular endothelial cell remains to be clearly established. In the present study, we have investigated the role of PKC in acute clofibrate (PPARα-ligand) α-induced regulation a of NO production and PPAR expression in cerebral microvascular endothelial cell culture. Our data reveal complex interactions between PKC and NO production as well as PPARexpression following PPAR activation.

Materials and methods

Chemicals

Reagents used in the present study were obtained from the following companies: clofibrate, L-arginine methyl ester (L-NAME), endothelial growth supplement, Percoll, Dulbecco's minimum essential medium (DMEM) (Sigma- Aldrich, St Louis, MO), calphostin C, phorbol 12-myristate 13-acetate (PMA) (Calbiochem, San Diego, CA), polyclonal antibodies for PPAR and eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), Matrigel and Cell culture plates (BD Biosciences, San Jose, CA), Fetal bovine serum (FBS) (Atlanta Biologicals, Norcross GA).

Primary cultures of cerebral microvascular endothelial cells:

Primary culture of cerebral microvascular endothelial cells from pig brain was established as previously described (13). Briefly, cerebral cortical microvessels (60–300 μm) were isolated by differential filtration of cerebral cortex homogenate, first through 300-μm and then through 60-μm nylon mesh screens. The isolated microvessels were incubated in collagenase-dispase solution (1 mg/ml) for 2 h at 37 °C. At the end of the incubation, the dispersed microvascular endothelial cells were separated using Percoll density gradient centrifugation. Endothelial cells were collected, washed, and resuspended in culture medium consisting of 20 % fetal bovine serum (FBS), 2 mg/ml sodium bicarbonate, 1 U/ml heparin, 30 mg/ml endothelial cell growth supplement, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B, the cells plated on 12-well Costar plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ), and maintained in a 5 % CO_2-95 % air incubator at 37 °C. The culture medium was changed every 2–3 days until the cells attained confluence. Confluent cells (after 7–10 days of cultivation) were starved overnight with 1 % FBS conditioned media and used for the experiments.

Incubation of cells with vasoactive agents:

To investigate the roles of PPARα, PKC, or NOS in NO production, cells were incubated with clofibrate (10 μM), a PPAR α activator, phorbol-myristate acetate (PMA) (1 μM), a PKC activator, calphostin C (1 μM), or L-NG-nitro-arginine methyl ester (L-NAME) (0.1 mM), an inhibitor or NOS inhibitor, alone or in combination for 6 hr. At the end of the incubation, media was collected for determination of NO production. In addition, cells were lysed by lysis buffer containing 2.5 % SDS, 0.125 M Tris-HCl, 10 % glycerol, and 5 % β-mercaptoethanol and collected for protein estimation and western blot analysis. Protein content of the samples was estimated by the Bradford method.

Griess Assay:

NO concentration in the media was determined using the Griess Assay. Briefly, assay samples were mixed with an equal volume of the Griess reagent [0.1 % *N* (1-naphthyl) ethylenediamine dihydrochloride and 1 % sulfanilamide in 3 % H_3PO_4) and incubated at room temperature to yield a chromophore. Using Plate Reader (Model EL808UV, Bio-Tek Instruments, Uniooski, VT), the absorbance at 540 nm was measured, and nitrite concentration was determined using a nitrite standard curve. The efficiency was at least 95 %. Results were corrected for protein and expressed as nM/μg protein.

Western blot analysis:

To determine the effects of treatments on the regulation of eNOS and PPARα expression in cerebral mmicrovascular endothelial cells, 30 μg of total protein in Laemmli Sample Buffer was loaded per well and resolved on a 12 % SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane by the use of a wet transferring blotter for 2 h in a buffer containing 48 mM Tris-HCl (pH 8.5), 39 mM glycine, and 20 % methanol. The nitrocellulose was washed in Tris-buffered saline, blocked in 5 % nonfat dried milk in Tris-buffered saline (NFM/TBS) for 1 h, and incubated with a primary polyclonal rabbit anti- eNOS

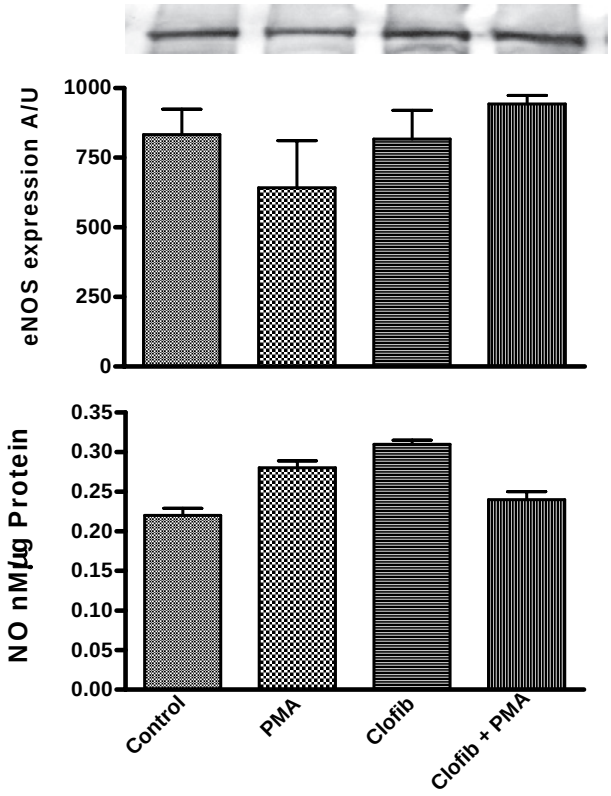

Fig. 1. Effect of PPARα activation on NO production and eNOS expression in CMVEC. Cells were treated with clofibrate (10 μM), PMA (1 μM), alone or combined for 6 h and NO determination by Griess assay and effects of treatments on eNOS protein expression were mdetermined by Western blot analysis of 30 μg protein per lane using specific eNOS antibody. Results of NO production was mcorrected for protein and expressed as nMol/μg protein with results from each duplicate set pooled and used as a data point. Results are presented as mean ± S.E.M. eNOS blot is a representative of 4 different blots and the results of the densitometric quantification of the bands presented as mean ± S.E.M. (* p<0.05, n=4 from different sets of cultures, ANOVA).

or -PPAR-α antibodies (1:500 dilution) in 5 % NFM/TBS overnight at 4 °C. The nitrocellulose was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies in 2 % NFM/TBS for 2 h. The bound antibody was detected by enhanced chemiluminescences (ECL, Santa Cruz Biotech, Santa Cruz, CA) and the intensity of the bands was scanned and quantified using Personal Densitometer Scanner and ImaQuant analysis software (Molecular Dynamics, Sunnyvale, CA). Blots presented are representation of at least 3 of such blots.

Statistical analysis:

Data are presented as mean ± SEM; differences between groups were assessed using one-way ANOVA followed by Turkey comparison tests. A value of $p<0.05$ was considered significant. In the determination of effects of treatment of the cells on NO production, cells were treated in duplicate, NO determination in each sample was carried out in duplicate and the results were combined to represent a data point. The n value reported represents number of separate experiments from different cultures.

Results

Effects of PMA or Clofibrate on NO production and eNOS expression

Basal production of NO in cultured cell was 0.22±0.01 nM/μg protein. Figure 1 (lower panel) indicates that treatment of these cells with clofibrate (10^{-5} M), a PPARα agonist, for 6 hrs resulted in an increased release of NO into the cultured media (41±4.5 %, $p<0.01$, n=4). Similarly, treatment of CMVEC with PMA (10^{-6} M), a PKC activator, for 6 hrs increased NO production by 27±6 % ($p<0.001$, n=4). However, there was no change in the amount of NO released into the culture media following treatment of CMVEC with combined PMA and clofibrate at the same concentration and for the same duration of treatment as above (0.24±0.01 versus 0.22±0.01 nM/μg protein). In order to determine whether PPARα activation or PKC stimulation-induced increase in NO production, hence, increased NOS activity is associated with posttranscriptional effects, we evaluated the effects of clofibrate or PMA on the expression of eNOS protein. Neither clofibrate nor PMA or combined treatment of CMVEC with both agents had an effect on eNOS expression at the same concentrations and treatment periods that produced increased NO production (Fig. 1, upper panel: n=4, ANOVA, $p<0.05$).

Effect of PKC inhibition on NO production and eNOS expression

As PKC is known to be involved in NO synthesis, we evaluated the effects of inhibition of PKC on basal and clofibrate-induced increase in NO production. Figure 2A illustrates the effect of pretreatment of cells for 15 min with calphostin C (1 μM), a PKC inhibitor, on the amount of NO released into the media under basal condition and when CMVEC were challenged with PMA, a PKC activator. Calphostin C reduced basal NO production from 0.22±0.01 to 0.19±0.001 nM/μg protein. Moreover, calphostin C abolished PMA-induced increase in NO production reducing NO level from 0.28±0.03 to 0.21±0.02 nM/μg protein. However, inhibition of PKC with calphostin C did not affect clofibrate-induced increase in NO production {(0.31±0.004; clofibrate, versus 0.31±0.005; Clofibrate + Calphostin C)} nM/μg protein, n=4, ANOVA, $p<0.05$; Figure 2B. As noted in Figure 1, eNOS protein expression was not affected by any of these treatments.

Effect of NOS inhibition on clofibrate-induced NO production

As increased NO production could reflect increased NOS activity, we determined NO released into the cultured media following treatment with clofibrate in cells pre-incubated with L-NAME, a NOS inhibitor. Figure 3 illustrates that L-NAME (10^{-4} M) reduced basal NO production by 47 % (ANOVA, $p<0.001$, n=5). In addition, L-NAME blunted clofibrate-induced increase in NO by 20±1 % ($p<0.05$, n=5) indicating that clofibrate increased NO production in CMVEC via increase in NOS activity ($p<0.05$, ANOVA, n=5) (Fig. 3).

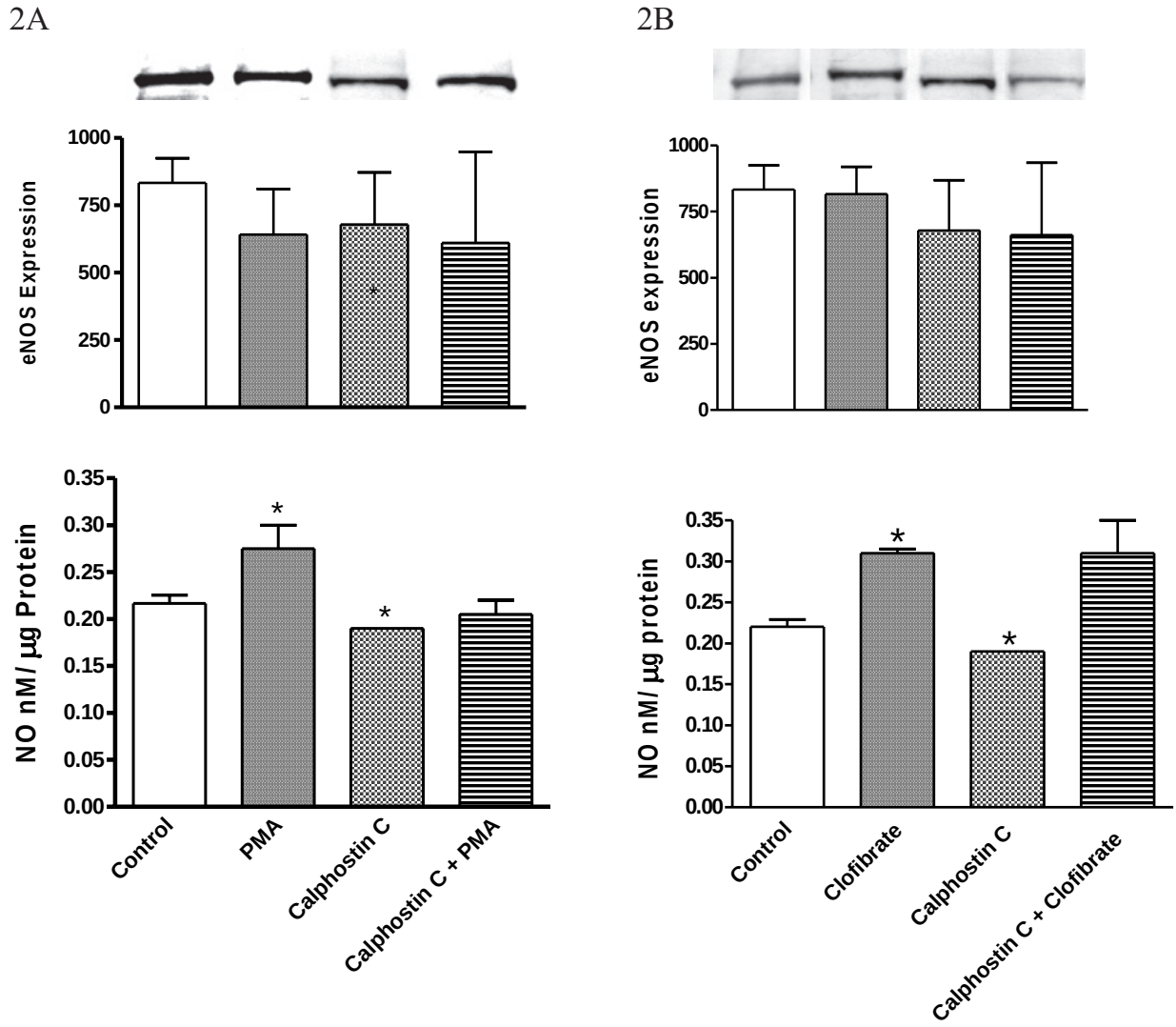

Fig. 2. Role of PKC in NO production and eNOS expression by CMVEC. To inhibit PKC, cells were incubated with Calphostin C (1 µM) for 15 m min before exposing it to PMA (1 µM) (Fig. 2A) or clofibrate (10 µM) (Fig. 2B) and incubated for a further 6 h. At the end of the incubation, media were collected for NO determination by Griess assay and effects of treatments on eNOS protein expression were determined by Western blot analysis of 30 µg protein per lane using specific eNOS antibody. Results of NO determination were corrected for protein and expressed as nM/µg protein, results from each duplicate sets were pooled and used as a data point presented as mean ± S.E.M. The eNOS blot is a representative of 4 different blots and the results of the densitometric quantification of the bands presented mean ± S.E.M. (* p<0.05, n=4 from different sets of cultures, ANOVA).

Effect of PKC inhibition on PPARα expression

Incubation of endothelial cells with PPARα activator, clofibrate (10 µM) increased PPARα expression by 30 % ($p<0.001$). Treatment with PMA (1 µM) PKC activator or calphostin C (10 µM) PKC inhibitor, significantly reduced basal PPARα expression. The reduction in PPARα expression induced by PMA was 40 % ($p<0.01$) while the reduction in the presence of PKC inhibitor, calphostin C was 90 % ($p<0.001$) of the basal (control) value. PMA markedly blunted clofibrate-induced increase in expression of PPARα by 92 % ($p<0.001$). Similarly, PKC inhibition by calphostin C reduced basal PPARα expression by 89 % ($p<0.001$) and reduced clofibrate-induced increased expression by 65 % ($p<0.001$; ANOVA, n=3) (Fig. 4).

Discussion

We have investigated the role of PKC in acute activation of PPARα-induced increase in NO production and have found that (1) treatment of CMVEC with PMA or clofibrate alone but not the combination increased NO level without affecting eNOS expression; (2) inhibition of PKC reduced basal NO and abrogated PMA-but not clofibrate-induced increase in NO; (3) L-NAME

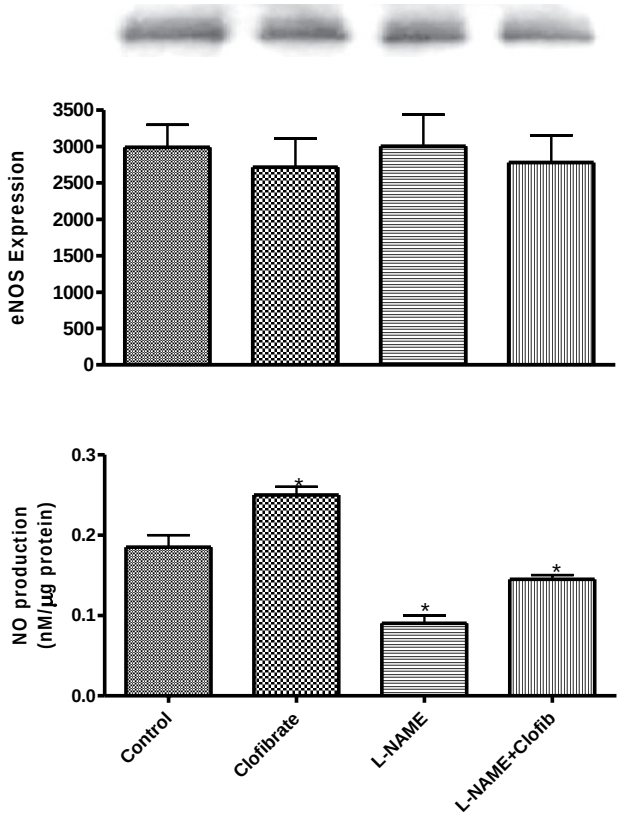

Fig. 3. Role of NOS in acute clofibrate-induced NO production. Effects of inhibition of eNOS with L-NAME (0.1 mM) on clofibrate (10 μM)-induced increase in NO production was determined in CMVEC. Cells were incubated with L-NAME for 15 min before exposing it to clofibrate for further 6 h incubation. At the end of the incubation, media NO was determined by Griess assay and effects of treatments on eNOS protein expression were determined by mWestern blot analysis of 30 μg protein per lane using specific eNOS antibody. Results of NO determination were corrected for mprotein and expressed as nM/μg protein, results from each duplicate sets were pooled and used as a data point, and presented as mean ± S.E.M. The eNOS blot is a representative of 4 different blots and the results of the densitometric quantification of the bands presented as mean ± S.E.M. (* p<0.05, n=4 from different sets of cultures, ANOVA).

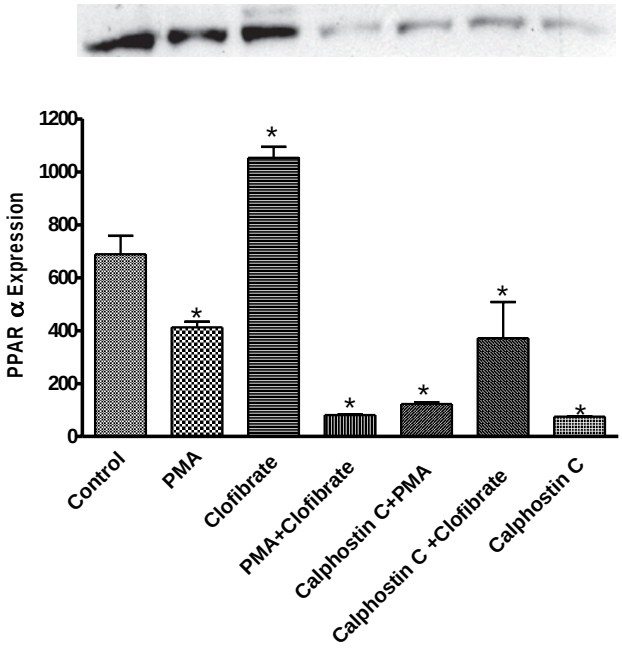

Fig. 4. Role of PKC in the regulation of PPARα expression in CMVEC. Effects of incubation of CMVEC with PMA (1 M), clofibrate (10 μM), or calphostin C (1 μM) alone or following inhibition of PKC (calphostin C, (1 μM) on the PPAR expression were determined. PKC inhibition was achieved by 15 min incubation of the cells with calphostin C before addition of PMA or clofibrate for further 6 h incubation. At the end of the incubation, lysed cells were acollected and effects of treatments on PPARα expression were determined by Western blot analysis of 30 μg protein per lane aausing specific PPARα antibody. The PPARα blot is a representative of 3 different blots and the results of the densitometric quantification of the bands presented mean ± S.E.M. (* p<0.05, n=3 from different sets of cultures, ANOVA).

attenuated basal and clofibrate-induced increase in NO; (4) clofibrate-induced increase in PPARα expression was attenuated by both PKC activation and inhibition, and (5) PMA-induced reduction in basal PPARα expression was further reduced by PKC inhibition. These results show that in the brain microvascular aendothelial cell, PPAR activation increased NO production via a a mechanism independent of PKC signaling pathway and regulation of PPARexpression is mediated through a dual-complex PKC signaling pathway both PKC activator and inhibitor reduced PPARα expression.

The integrity of the vascular endothelial cell is fundamental to the normal functioning of the vascular system. In various disease states such as atherosclerosis or diabetes, multiple or individual risk factors can alter vascular homeostasis through dysregulation of signal transduction pathways, such as NO often resulting from activation of PKC. PKC activation has been reported to mediate many cellular functions including regulation of cellular proliferation and differentiation (11), generation of reactive oxygen species (22), modulation of synthesis and release of vasoactive agents (13, 23, 24). Enhanced PKC activation has been reported to be responsible for several vascular disease conditions and has been linked to vascular dysfunction (22–26). Vascular dysfunction resulting from the loss of NO dilatory mechanism leads to a state of heightened vascular tone and oxidative stress (1, 25, 26). In recent studies, PPAR activation has been reported to ameliorate vascular disease conditions characterized by reduced NO production and enhanced PKC activation (4, 5, 17, 27). The mechanism by which PPAR activation restores vascular function is not clear. In the present study, treatment with clofibrate, a PPARα activator, increased NO release into the culture media – a finding consistent with suggestions that PPAR induced vasoprotection could be due to improved NO production. The increase in NO production level may be responsible for the restoration of normal vascular function reported following administration of PPAR activators (28, 29). Increased NO

production can potentially result from many mechanisms including but not limited to increased NO synthase activity, increased substrate and or cofactors, and prevention of NO degradation. Increased NOS activity and/or expression is naturally a starting point for evaluating the mechanism involved in PPARα ligand-induced increase in NO production. We therefore evaluated the possibility of changes in eNOS expression. However, the increased NO production following treatment with PPARα activation does not seem to be due to increased eNOS expression, as expression was not changed following clofibrate, leading us to the conclusion that the acute effects of PPAR activation probably did not account for increased NO production. However, it appears that the source of increased NO production observed in the present study resulted from increased eNOS activity in as much as inhibition of NOS by L-NAME resulted in reduction of basal as well as PPARα-induced increase in NO production. Consistent with this notion is the reported increase in eNOS activity following treatment of bovine endothelial cell awith fenofibrate (30). PPAR ligands are known to possess antioxidant actions and to preserve endothelial-derived NO production (31). Reactive oxygen species can attenuate NO production, therefore, the increased NO production in the present study may be accounted for by the ability of PPAR to restore or preserve eNOS-derived NO in the brain endothelial cell via it antioxidant property – a fact that may account for PPAR activation induced amelioration of vascular dysfunction.

PKC, an ubiquitous signaling protein with several isoforms is responsible for the mediation of actions of various vasoactive, agents and has been implicated in the regulation of PPAR-activation, NO production, and in vascular dysfunction (15, 20, 21, 32). The role of PKC in the regulation of PPARα-induced production of NO was investigated and we found that PKC activator, PMA, significantly increased NO production and the inhibitor calphostin C reduced it indicating that PMA acts via PKC to regulate NO production. However, neither PKC activation nor inhibition had any significant effects on clofibrate-induced NO production suggesting that PKC may not be involved in acute PPARα activation induced increase in NO production. Furthermore, the role of PKC in clofibrate-induced PPARα expression was evaluated and our data demonstrated that clofibrate-induced increase in PPARα expression was significantly attenuated in the presence of both PKC activator and inhibitor. This result is surprising and the reason behind this observation is not known but it may be due to the sensitivity of PKC isoform(s) that may abe involved in the regulation of PPAR expression to PMA or calphostin C. However, others have demonstrated complex regulation of PPAR activation by PKC. For example, Blanquart et al (20), demonstrated that PKC may control PPAR activity by regulating its expression level as well as the response to its ligands in hepatocyte (HepG2 and HuH-7 cell lines) leading to the conclusion that PKC signaling pathway acts as a molecular switch dissociating the transactivation and transrexpression functions aof PPAR involving phosporylation processes. Consistent with this observation is the reported dual role of PKC in the aregulation of PPAR expression following chronic activation with clofibrate in cerebral microvascular endothelial cells (15). By contrast, Yaacob et al (32) reported that PKC signal transduction pathway contributes to the regulation of PPARα expression but does not influence the transcriptional activity of PPARα. These results along with others have shown that the relationship between ligand-induced PPAR expression and/or function and PKC signaling pathways is complex and needs further investigation to identify the specific PKC pathway(s) and aligands through which PPAR expression and/or functions are modulated.

In conclusion, PPARα activation induced increase in eNOS-derived NO production via a possible increase in NOS activity independent of PKC signaling pathways, but clofibrate induced PPARα expression was regulated by a complex process involving PKC activation and inhibition. The precise PKC isoform and pathways that are required for the regulation of PPARα activation and activity needs to be further investigated along with the physiological factors that may be involved in its modulation.

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