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Involvement of PGE2 and PGDH but not COX-2 in thrombin-induced cortical neuron apoptosis

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Abstract

The pathways that contribute to thrombin-induced neuron death have been incompletely defined. Induction of cyclooxygenase 2 (COX-2), the enzyme that catalyzes the first step in prostaglandin synthesis, promotes neuronal injury. PGE2, a downstream product of COX-2 metabolism, is neurotoxic *in vitro* and *in vivo*, and is thought to be the bioactive mediator responsible for COX-2 neurotoxicity. The objective of this study is to determine the ability of thrombin to affect PGE2 metabolism in cultured neurons. The data show that in thrombin-induced apoptosis of cultured neurons, PGE2 release increases when COX-2 is absent, and is regulated by prostaglandin dehydrogenase (PGDH), a key enzyme that degrades PGE2. NS398, a COX-2 specific inhibitor, protects neurons against thrombin toxicity, by inducing active PGDH. These data implicate PGDH in thrombin-mediated neuronal cell death.

Keywords

COX-2; PGE2; neurons; and thrombin

Introduction

Neuronal loss is a critical feature of brain injury and neurodegenerative diseases such as Alzheimer's disease (AD). The multi-functional serine protease thrombin has been shown to be increased in AD and other neuropathological conditions and is thought to contribute to pathological neuronal death [1,2,17,18,30,32]. Thrombin has been shown to cause memory loss *in vivo* as well as neuronal cell death *in vivo* and *in vitro*, [8,9,10,13,31,32,40,44]. The pathways and mediators that contribute to thrombin-induced neuron death have been incompletely defined. In primary cultured neurons we have shown that thrombin-induced apoptosis proceeds via cell cycle activation involving cyclin dependent kinase 4 and expression of the pro-apoptotic protein Bim [33]. In non-neuronal cell types, thrombin induces expression of cyclooxygenase 2 (COX-2) and prostaglandin (PG) PGE2 release [21].

Epidemiologic studies demonstrate that chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) in normal aging populations reduces the risk of developing AD. NSAIDs inhibit the enzymatic activity of COX-1 and inducible COX-2 which catalyze the first committed step in the synthesis of prostaglandins [46]. Induction of COX-2 expression and enzymatic activity promotes neuronal injury while inhibition of COX-2 activity is neuroprotective in a number

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of rodent models of neurological disease [3,12,19,23,24,28,35]. PGE2, a downstream product of COX-2, is neurotoxic both *in vitro* and *in vivo*, and is thought to be the bioactive mediator responsible for COX-2 neurotoxicity [5,14,22,27]. In this regard, COX-2 specific inhibitors such as NS398, which decrease PGE2 release, exert neuroprotective effects. Little is known about the regulation of prostaglandin dehydrogenase (PGDH) in the CNS, the enzyme responsible for PGE2 inactivation, although down regulation of this enzyme has been documented in response to inflammatory mediators [26,43]. The effect of the neurotoxic, inflammatory protein thrombin on COX-2, PGE2 and PGDH in neurons is unknown. Therefore, the objective of this study is to determine the ability of thrombin to affect PGE2 metabolism in cultured neurons and whether neuroprotection by NS398 is dependent on COX-2 inhibition.

Material and Methods

Primary neuronal cultures

Rat cerebral cortical cultures were prepared from 17-day gestation rat fetuses, as previously described [16,34]. The cells were plated at a density of 500,000/ml on multi-well plates coated with poly-L-lysine, in media containing DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated horse serum, and antibiotic/antimycotic. On day 3, the medium was changed to Neurobasal medium containing B-27 supplement, antibiotic/antimycotic, and 0.5 mM glutamine, and 20 μg/ml 5-fluoro-2′-deoxyuridine to inhibit proliferation of non-neuronal cells. On day 6, fresh medium without 5-fluoro-2′ deoxyuridine was used to replace the old media. Experimental treatments were carried out on day 8 in Neurobasal medium containing N-2 supplement, 0.5 mM L-glutamine and antibiotic/antimycotic (treatment medium).

Treatment of neuronal cultures with thrombin

Neurons were rinsed with HBSS and then treated with thrombin or NS398 in various combinations, in Neurobasal medium containing N-2 supplement and 0.5 mM L-glutamine and antibiotic/antimycotic (treatment medium). For the dose experiment, neurons were treated with thrombin (25 – 300 nM), for 24 h in treatment medium. Pre-treatment for 20 minutes with 100 μM NS398 in treatment medium was followed by addition of 100 nM thrombin to the neurons for 3 h. Supernatants and protein from control and treated cells were stored at −80°C.

Western blotting

Neurons were treated as described above, rinsed with HBSS and total protein was extracted from the neurons using lysis buffer containing 0.1% SDS, 1% Triton X-100 and 0.5% phenylmethyl sulfonylfluoride. Protein was estimated by the Bradford method using Bio-Rad protein reagents. Equal amounts of protein were resolved on a 10% polyacrylamide gel, transferred on to a PVDF membrane, blocked with 5 % milk solution (non-fat dry milk in TBST) and immuno-blotted with primary antibodies for COX-2 – 1:1000 (160116; Cayman Chemical, Ann Arbor, MI), PGDH – 1:1000 (NB200-179; Novus Biologicals, Littleton, CO) and GAPDH – 1:1000 (MAB374; Chemicon, Temecula, CA) and peroxidase-conjugated secondary antibodies. Membranes were developed with chemiluminescence reagents and exposed for equal times. Band intensities were quantified using the Quantity-One software (BioRad) and expressed graphically as intensity units, which is the average intensity over the area of a band.

Neuronal viability (XTT) assay

The viability of neurons under various treatment conditions was assayed using the cell counting kit-8 (CCK-8). The reagent contains a tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt),

which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 solution was added directly to the cell media for 30 min and supernatants were colorimetrically assayed at 450 nm. The amount of orange formazan produced was directly proportional to the number of living cells; this was assayed by measuring the absorbance of the media of cells incubated with CCK-8 reagent, against a blank (CCK-8 reagent in fresh media). The absorbance from untreated cells (controls) was set to 100%.

PGE2 assay

PGE2 release from neurons was assayed by ELISA using a kit from Designs, Ann Arbor, MI, USA. Samples and standards were incubated for 2 h at room temperature, followed by addition of substrate and a 45 min incubation at room temperature. After this, enzyme action was stopped and the green color generated read on a microplate reader at 405 nm. The intensity of the bound green color was inversely proportional to the concentration of PGE2 in either standards or samples. The measured optical density was used to calculate the concentration of PGE2 in the samples.

Statistical analysis

Results are expressed as means ± SD. Pairwise comparisons between controls and treatment groups were conducted using the one-way ANOVA followed by the Tukey test for multiple comparisons. $P<0.05$ was considered significant.

Results

Thrombin induces COX-2 expression in cultured neurons

Neurons were treated with a range of thrombin concentrations for 24 h, and examined for the expression of COX-2 protein. Western blot analysis showed a faintly detectable band at 72 kD in neuronal cultures treated with 100 nM thrombin (Fig. 1). Untreated cells and neurons exposed to 25 to 50 nM thrombin did not express measurable COX-2. In contrast, treatment of neurons with 200nM or 300 nM thrombin evoked intense staining for COX-2.

We have previously shown that thrombin-treated neurons commit to apoptosis within 3 h following treatment [33]. It is of interest to note that there was no expression of COX-2 in the thrombin treated neurons until 16 h post exposure (data not shown). This suggests that induction of COX-2 may not be required for initiation of apoptosis in response to thrombin.

Thrombin causes neuronal cell death and increased PGE2 levels at 3 h

Exposure of neurons to 100 nM thrombin for 3 h caused a significant ($p<0.001$) decrease in neuronal survival (52%) compared to untreated neuronal cells (100%).

Based on observations that implicate PGE2 as a relevant mediator of neurotoxicity we examined the ability of thrombin to affect PGE2 levels in cultured neurons. Neurons were treated with 100 nM thrombin for 3 h, supernatants collected and used to measure PGE2 by ELISA. The data showed that thrombin treatment significantly ($p<0.05$) increased PGE2 levels in cultured neurons (Fig. 2)

NS398 decreases both thrombin-mediated cell death and PGE2 levels in neurons

Exposure of neurons to thrombin for 3 h resulted in a reduction in cell survival that was significantly ($p<0.001$) improved (52% to 83% survival), upon treatment with NS398. Also, treatment of neuronal cultures with NS398 significantly ($p<0.001$) diminished the increase in PGE2 evoked by thrombin treatment (Fig. 2). The ability of NS398 at 3 h to both decrease the cell death response to thrombin as well as the increase in thrombin-mediated PGE2 was not

due to inhibition of COX-2, because as indicated above there was no expression of COX-2 in cultured neurons at 3 h. The concentrations of NS398 used in the study are within the range of specific COX-2 inhibition, therefore, the possibility that other COX isoforms may be involved is unlikely.

NS398 regulates expression of the PGDH active form

Degradation of PGE2 is regulated by expression of the enzyme PDGH. PDGH exists in several forms (monomeric, dimeric or glycosylated) with the active forms in the 29 kD range and inactive forms migrating near 48 – 58 kD [11,15,36,37]. Neuronal cultures appear to express strong reactivity at both 48 kD and 29 kD (Fig. 3). Treatment of neurons with thrombin caused almost a complete disappearance of the higher migrating inactive form although evoking a slight increase in the active form. Pretreatment of neurons with NS398 prior to addition of thrombin increased expression of active form of PDGH compared to levels evoked by thrombin alone (Fig. 3). To our knowledge, this is the first report of PGDH expression in neurons.

Discussion

In this study, we sought to determine the involvement of COX-2 and PGE2, both of which are implicated in neurodegeneration, in the pathways of thrombin-mediated neuronal apoptosis. We have previously shown that thrombin-induced neuronal death proceeds through activation of cell cycle and pro-apoptotic proteins [33]. Therefore, in this study, we first determined whether expression of COX-2 and PGE2 preceded cell cycle activation or not. Surprisingly, expression of COX-2 was not evident until 24 h, long after cell cycle proteins would be activated under the same conditions. Yet, PGE2 release was significantly higher in thrombin-treated neurons compared to untreated controls within 3 h, corresponding with earlier results of cell cycle and apoptosis activation. Pre-treatment with NS398, a COX-2 selective inhibitor, significantly ($p<0.001$) increased neuronal survival, and also blocked the increase in PGE2 in control as well as thrombin-treated neurons, although COX-2 expression was absent at that time point. Therefore, it was apparent that the mechanism of PGE2 regulation was not by COX-2 inhibition.

Although NSAIDs, such as NS398, inhibit prostaglandin synthesis enzymes, COX-1 and 2, it is possible that these enzymes are not their only targets. Studies have suggested that the antitumor effects of NSAIDs are mediated via a COX-2-independent mechanism. In this regard, NSAID-mediated inhibition of glioma proliferation depends on regulation of PGDH, a key prostaglandin catabolic enzyme [47]. The results of the current study support the notion that NSAIDS may, in part, affect PGE2 by changes in the activity of PGDH. We show that the expression of active PGDH increases in neurons treated with NS398, both alone as well as in the presence of thrombin (Fig. 3). In neurons, it appears that NS398 induction of active PGDH is transient and therefore neuroprotection mediated by NS398 via PGE2 decrease is also not long lasting. In contrast, thrombin can elicit both acute (3 h) and longer term (24 h) toxicity because it likely targets multiple pathways/mediators such as PGE2, COX-2 as well as cell cycle proteins [33].

In a hypoxia model of neuronal injury where COX-2 is responsible for neurotoxicity, it has been demonstrated that overexpression of COX-2 did not increase expression of cyclin D1, or phosphoretinoblastoma protein (pRb), or cleavage of caspase 3, suggesting that a cell cycle-dependent mechanism does not mediate COX-2 toxicity in this model [25]. Therefore, it appears that cell cycle-mediated neuronal apoptosis and COX-2 mediated apoptosis may be independently initiated pathways that have certain events, such as PGE2 regulation, in common. The COX-2 pathway may also be a later, fail-safe mechanism to eliminate neurons that are stressed but have not committed to apoptosis via cell cycle activation. Results from a study that examined thrombin-mediated (via PAR1) increase in PGE2 in rat gastric mucosal

cells show that the increase was suppressed by inhibitors of COX-1, COX-2 MEK, p38, MAP kinase, and protein kinase C [38]. In that study the authors conclude that thrombin stimulates complex multiple signaling pathways responsible for PGE2 formation, it is possible that the results from the current study reflect this complexity in neurons.

Influx of calcium is widely recognized as an inducer of prostaglandin output [6]. Thrombin has been demonstrated to cause a rapid influx of calcium in neurons and neuronal cell lines and this increase is thought to induce neuronal degeneration [4,20,29,39,41,42,45]. Influx of calcium in nonneuronal cells suppresses expression of PGDH and enhances PGE2 production [6]. In the context of our data, thrombin-induced disappearance of the PGDH inactive form may be correlated to calcium elevation. Treatment with NS398 is reported to significantly decrease calcium influx in hippocampal dentate granule neurons [7]. Therefore in our study, although NS398 may be able to partially counter the calcium influx, and sustain expression of active PGDH, it appears to be transient. In this regard, NS398 was able to increase neuronal survival in response to thrombin (from 52% to 83%) at 3 h but was ineffective as a neuroprotectant at 24 h (data not shown). The necessity for long-term administration of NSAIDs might be a consequence of the relatively short duration of their effect on activation of PGDH, resulting in PGE2 degradation. Further studies on long-term use of agents such as NS398 could shed light on the importance of PGE2 degradation via PGDH in mediating neuronal cell death by thrombin and other neurotoxins. The appearance of COX-2 at 24 h after thrombin treatment suggests an additional late-phase inflammatory pathway in neurons. Thus, thrombin-mediated neuronal apoptosis likely involves more than one pathway, and may not easily be reversed by single target therapeutics.

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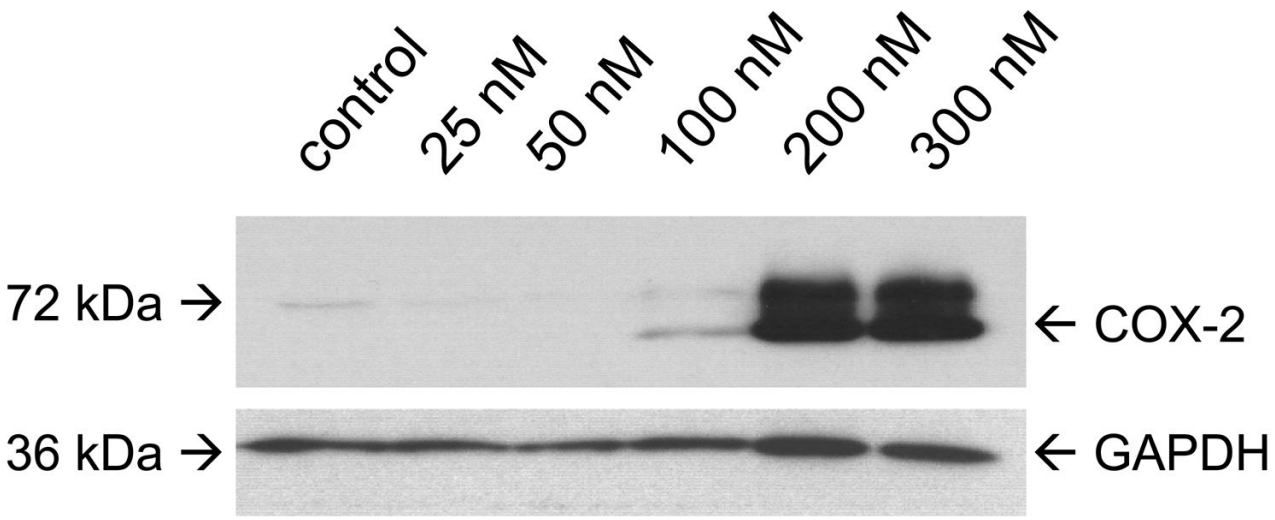

Figure 1.
Western blot analysis of COX-2 expression in cortical neurons induced by thrombin. Cultured neurons were exposed to a range of thrombin concentrations (0–300 nM) for 24 h. Cells were rinsed and lysed and lysates (25 μg) run on SDS-PAGE gel and western blot analysis performed using antibodies to thrombin. Antibodies to GAPDH were used to confirm loading equivalency between lanes. The blot is representative of 3 separate experiments.

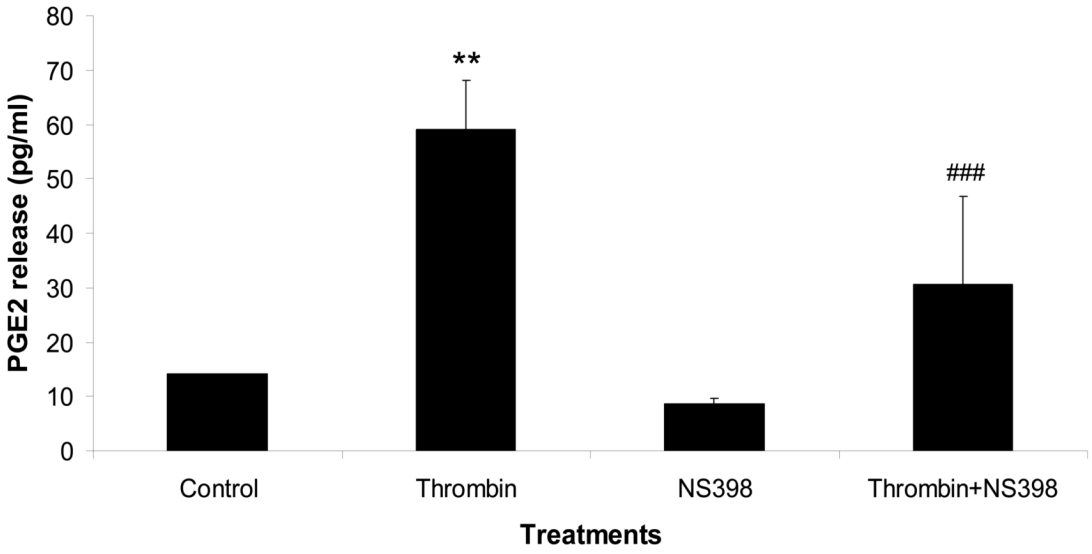

Figure 2.

Effect of thrombin and NS398 on PGE2 levels in cultured neurons. Neurons in treatment media were treated with thrombin (100 nM), NS398 (100 μM) or both and incubated for 3 h, the media were then collected and analyzed for PGE2 by ELISA. The results are means ± SEM for 3 experiments performed in triplicate.

** $p<0.01$vs.control

$p<0.05$ vs. thrombin alone

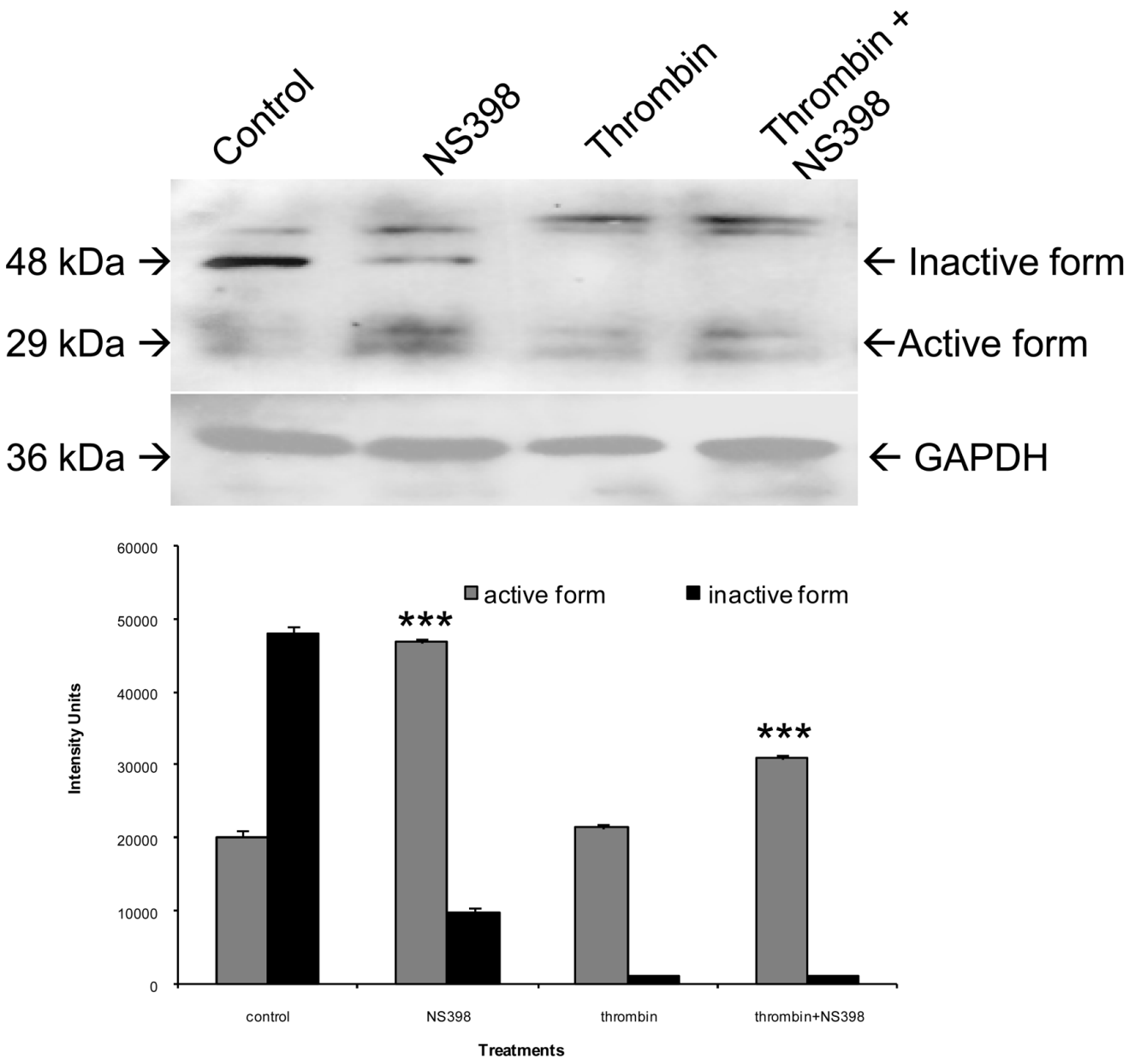

Figure 3.

Western blot analysis of PGDH in neurons regulated by NS398 and thrombin. Cultured neurons in treatment media were treated with thrombin (100 nM), NS398 (100 μM) or both for 2 h, protein lysed and analyzed by western blotting for Prostaglandin Dehydrogenase (PGDH). The housekeeping gene GAPDH was used as a loading control. Results are means ± SEM for 3 experiments. ***$p<0.001$ vs control; n=3.