ENDOTHELIAL EICOSANOID METABOLISM AND SIGNAL TRANSDUCTION DURING EXPOSURE TO OXYGEN RADICALS INJURY

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Abstract  Several physiological agonists that induce elevation of cytosolic free calcium (Ca2+)-levels act via receptor coupled G-proteins, involving activation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4,5-bisphosphate. Activation of the inositol signal transduction pathway that precedes Ca2+ ion mobilization is a well accepted signaling pathway in endothelial cell eicosanoid synthesis. This study was designed to examine possible involvement of phosphoinositides in the effects of oxygen free radicals on Ca2+ liberation and eicosanoid synthesis in human umbilical venous endothelial cells (HUVEC). Hydrogen peroxide (H2O2) was chosen as oxygen radicals generating agent. Stimulation of HUVEC with H2O2 (0.1 mmol/l) led to significant rises in inositol phosphate and diacylglycerol (DAG) levels within 300 seconds and an inhibition of Ca2+ release from internal stores. Eicosanoid formation was detectable despite unchanged levels of cytosolic free Ca2+ and no detectable activation of membrane associated phospholipase A2 (PLA2). This suggests that eicosanoid formation may be mediated through the activation of a Ca2+ independent, cytosolic 40 kDa PLA2 isoenzyme and that DAG could serve as an alternative source for arachidonic acid and seems to sensitize a cytosolic PLA2.

Key words: Endothelial cells, oxygen radicals, eicosanoids, signal transduction
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It is well accepted that various agonists activate phospholipase C (PLC) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to yield inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (1). IP3 specifically binds to the endoplasmic reticulum, initiating the rapid release of Ca2+ which leads to the activation of Ca2+-dependent enzymes such as PLA2 resulting in subsequent arachidonate release. PLA2, a lipolytic enzyme that specifically hydrolyzes the sn-2 ester bond of phospholipids, is present in a number of cells including endothelial cells. In endothelial cells, the PLA2 selective for arachidonic acid release is thought to be a 85 kDa enzyme (2). Diacylglycerol (DAG), a compound generated by phospholipase C mediated phosphoinositide hydrolysis, is also able to stimulate PLA2 activity by decreasing the packing density of the lipid bilayer, thereby increasing substrate accessibility to the enzyme (3).

PLA2 action on membrane lipids represents an important pathway for arachidonic acid release. Mayer and Marshall (2) described two major routes for the control of arachidonic acid mobilization: PLA2 activation through a PLC-mediated pathway with activation of PKC and elevation in Ca2+ levels, and a PLC independent activation via a G-protein. An alternative source for the arachidonate required for eicosanoid synthesis is DAG (4), which activates protein kinase C (PKC). PKC, which inhibits PLC through a negative feedback mechanism, can also be selectively activated by phorbol 12-myristate 13-acetate (PMA), an analogue of DAG. In a previous study we were able to demonstrate, that addition of PMA to endothelial cells led to a strong and reversible rise in free Ca2+ but failed to stimulate eicosanoid production (5). Thrombin-stimulated eicosanoid production was not affected by PMA, whereas ATP induced synthesis was strongly inhibited. Jaffe et al. (6) demonstrated that thrombin stimulation of HUVEC induces rapid, coordinate rises in IP3 and Ca2+ levels and elevation of prostaglandin I2 (prostacyclin; PGI2) production. They suggested that IP3 acts as the second messenger through which thrombin elevates Ca2+ and initiates PGI2 synthesis.

Since it has been demonstrated that oxygen radicals derived from hydrogen peroxide induce eicosanoid formation in HUVEC (7-10) and lead to changes in Ca2+ signaling (11-13), the present study focuses on the association between the onset of oxidative stress and the activation of the phosphoinositide signal transduction pathway. The molecular mechanisms responsible for oxidant-induced endothelial cell damage remain largely unknown. Boyer et al. postulated that hydrogen peroxide-stimulated arachidonic acid release is not mediated via calcium but that a stimulation of intracellular kinase activity is necessary for this response (14). Furthermore, Stauble et al. have clearly demonstrated that the effect H2O2 has on PKC is similar to PMA (15).

To investigate whether oxygen radicals induced eicosanoid synthesis is also mediated via activation of PLC with subsequent IP3 formation or whether direct activation of PLA2 occurs, the intracellular formation of inositol phosphates as well as the activity of membrane associated PLA2 was measured. Furthermore, a comparison between the physiologically relevant substance thrombin and reactive oxygen species with respect to peak levels and time courses of inositophosphates was performed to help shed light on the possible biological significance of the inositol signal transduction pathway in the deleterious action of oxygen radicals.

MATERIALS AND METHODS

Cell isolation and culture.
Human umbilical veins were prepared using a modified standard procedure (5,7,16). Only cells from first subcultures were used for the experiments described below. The average cell density of all experiments performed on six different cell lines was 100575 ± 20444 cells/cm² (mean ±
Preparation of endothelial cell membranes.
Confluent primary cultures were washed twice with DPBS (Gibco, USA). Cells were harvested by gentle scraping, centrifuged at 800g, resuspended, split 1:5 and seeded into T-75 culture flasks. Cells were cultivated and identified as endothelial cells as described above. Confluent endothelial cells from the first subculture were washed twice and scraped in presence of DPBS. Cells originating from the same primary culture were pooled, thereafter centrifugation was performed at 200x g for 5 minutes. The supernatant was decanted and cells were resuspended in 5 ml tris(hydroxymethyl) aminomethane (TRIS)/HCl (50 mmol/l, pH 7.4) and frozen immediately at - 80° C. After thawing, cells were homogenized (for 30 sec., three times) using a polytron (Ultraturrax T25; Janke and Kunkel, Germany). During homogenization the vial was kept on crushed ice. Thereafter the homogenate was centrifuged at 3500x g for 10 minutes to remove the nuclei. The supernatant was transferred into Beckman high speed vials and centrifuged at 40000 x g for 20 minutes (4° C). After removing the supernatant the membranes were resuspended in 500 µl TRIS/HCl (50 mmol/l, pH 9.0) containing 100 µmol/l CaCl2. Aliquots of 100 µl were stored at -80° C. The membrane suspension will be referred as MS, the Tris/HCl/ CaCl2 buffer as THC.

Stimulation of PGI2 release.
For the stimulation experiments, the culture medium was removed and cell layers were gently washed with prewarmed (37° C) phosphate-buffered saline (PBS) and incubated with 1350 µl prewarmed (37° C) PBS containing 5 mmol/l glucose for one hour at 37° C. Cells were then stimulated for 5-600 seconds by the adding 150 µl of a solution containing one of the following substances: human thrombin (Sigma, USA), PMA (Sigma, USA) or H2O2 (Merck, Germany). The final concentrations yielded in the experiments were as follows: thrombin 1 U/ml; PMA 1 µmol/l; H2O2 0.1 mmol/l and 1.0 mmol/l. These concentrations of thrombin and H2O2 were chosen since they were proven to be potent stimulators of eicosanoid synthesis (7,16) and are of pathophysiological relevance (18). At the end of the incubation period the supernatants were transferred into micro test tubes containing indomethacin (30 µmol/l, final concentration) to avoid further formation of eicosanoids. Each experimental procedure was performed three times on six different cell lines. The supernatants were stored in presence of indomethacin at - 80° C.

Measurement of PGI2.
A direct radioimmunoassay (RIA; BIOTECX) was carried out to determine the concentration of 6-keto-prostaglandin F1α (6-keto-PGF1α), the stable degradation product of PGI2. There was no evidence of interference with the RIA by any of the substances used. The amount of PGI2 measured in the supernatants of unstimulated cells was approximately 40-fold higher than the detection limit (13.5 fmol/ml) of the RIA used. The specificity and reliability of the assay were confirmed by analyzing supernatants from stimulation experiments performed in presence of
the inhibitors indomethacin (cyclooxygenase) and OKY046 (TXA2 synthetase), as reported earlier (5,7,16). The percent cross reactivity of the antibody in the 6-keto-PGF1α-RIA was less than 0.5 % for PGE2. The intraassay coefficient of variation was 6.8 %, the interassay coefficient of variation was 10.9 %.

**Determination of intracellular free Ca2+**.

Ca2+ concentrations were determined and calculated as described elsewhere (19). After trypsinization of confluent monolayers of first subcultures, cell suspension was centrifuged for 10 minutes at 800g and the pellet resuspended in Hank's balanced salt solution (HBSS) buffer (140 mmol/l NaCl, 3.9 mmol/l KCl, 0.5 mmol/l Na2HPO4, 0.7 mmol/l KH2PO4, 11 mmol/l glucose, 0.5 mmol/l MgCl2, 2.2 mmol/l CaCl2, and 20 mmol/l N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)). Then Fura-2AM (Calbiochem Corp., USA) solution (1 mg in 1 ml dimethyl sulfoxide) was added to a final concentration of 10 µg/ml, and the cell suspension was incubated for 30 minutes at 37°C. After rinsing twice and centrifuging with HBSS buffer, the cell suspension was diluted to 6 x 10^6 cells/ml. Measurements were performed on 200 µl cell suspension in 2.3 ml prewarmed HBSS buffer (excitation: 342 nm; emission: 492 nm). Twenty-five microliters of a 100-fold-concentrated stimulant solution was added. Calibration was started by adding 50 µl EGTA solution (0.4 mol/l EGTA in 1.5 mol/l tris(hydroxymethyl)aminomethane (TRIS)). Thereafter cells were lysed by adding 25 µl Triton (10%(vol/vol) Triton X-100). FURA and EGTA were saturated with 50 µl 1 mol/l CaCl2 solution. The zero calibration was performed with HBSS buffer.

To assess whether trypsinization of the cells led to alterations in intracellular Ca2+ levels HUVEC were cultured on Cytodex microcarrier beads, a culture method facilitating the collection and subsequent analysis of cells without prior trypsinization. Both trypsinized cells and cells grown on beads were then analyzed and results obtained showed no differences in either percentage increase or time course profile of intracellular free Ca2+ levels. Furthermore, to exclude PMA- or H2O2-induced damage of the cell membrane leading to an artifactual Ca2+ influx, release of lactate dehydrogenase (LDH) was measured. Since there is a possibility of ion leakage prior to cell lysis the intracellular adenosine-5′-trisphosphate (ATP) content was measured. In an attempt to reverse ion loss, cells would increase the net inward flux, an energy consuming process which leads to reduced cellular ATP content. Both ATP and LDH remained unaltered under these experimental conditions compared with controls (data not given). Furthermore, in order to confirm cellular integrity, cells were examined by means of light and scanning electron microscopy.

**Measurement of Inositol Phosphates.**

HUVEC were cultured in six well culture plates and incubated for 24 h in growth medium containing myo-3H-inositol (15 µCi/ml) at 37°C. Cells were washed twice with PBS containing 5 mmol/l glucose and allowed to equilibrate for one hour. Thereafter, substances to be tested were added for time periods ranging from 5 to 600 seconds. The final concentrations yielded in the experiments were: thrombin 1 U/ml, PMA 1 µmol/l, and H2O2 0.1 and 1.0 mmol/l. At the end of the stimulation, cells were washed twice with DPBS without Ca2+ and Mg2+. The reaction was stopped by the addition of 50 mmol/l hydrochloric acid (HCl) and rapid freezing at -80°C. After thawing, the cells were scraped repeatedly and the labeled inositol metabolites were extracted with chloroform:methanol:HCl (3:3:2). Following centrifugation (5°C, 5 min., 3000 rpm), the 3H-labeled inositol phosphates were isolated by anion exchange chromatography using an AG1-X8 resin (200-400 mesh, formate form, Bio-Rad Laboratories, Richmond, USA) (20). 3H-IP1, 3H-IP2 and 3H-IP3 were eluted sequentially with increasing
concentrations of ammonium formate (0.1 - 1.0 mol/l). 2 ml fractions were collected in scintillation vials containing 18 ml Optiphase "HiSafe II" (LKB) cocktail and counted in a liquid scintillation beta-counter (Rackbeta, LKB). The recovery rates were 94-98% as assessed using 3H-inositol 1,4,5-trisphosphate, 3H-inositol 1,4-bisphosphate, 3H-inositol 1-monophosphate standards obtained from Amersham Radiochem.

**Stimulation and measurement of PLA2 in intact endothelial cells.**

HUVEC were cultured in six well culture plates and incubated for 24 h in growth medium containing [1-14C] arachidonic acid (0.25 µCi/ml) in a humidified incubator (37°C, 5 % CO2). Cells were washed twice with PBS containing 5 mmol/l glucose and allowed to equilibrate for one hour. Thereafter, substances to be tested were added for a time period of 300 seconds. The final concentrations yielded in the experiments were: thrombin 1 U/ml, H2O2 0.1 and 1.0 mmol/l. At the end of the stimulation, supernatants were withdrawn, transferred into glass tubes and extracted with equal amounts of chloroform, methanol and HCl (50 mmol/l). Cells were washed twice with DPBS without Ca2+ and Mg2+, lysed by adding 1 ml methanol/HCl (100:1, vol/vol) and rapid freezing at -80°C. After thawing, the cells were scraped repeatedly, transferred into glass tubes and extracted with equal amounts of chloroform, methanol and HCl (50 mmol/l). Following centrifugation (4°C, 5 min., 3000 rpm), the organic phases of the samples were evaporated under nitrogen stream and resuspended with chloroform:methanol (9:1, vol/vol). After mixing a 10 µl fraction was applied to a 60 F254 silica gel thin-layer chromatography glass plate (Merck, Germany) and developed in a solvent system of n-hexan:diethylether:glacial acetic acid 70:30:1 (vol/vol). After development of the chromatography plate the areas corresponding to free arachidonate were visualized by iodine staining, scraped out, transferred into a scintillation vial containing 10 ml of the scintillant Optiphase "High Safe II" (Pharmacia, Sweden) and the radioactivity was measured.

**Stimulation and measurement of PLA2 in endothelial cell membranes.**

90 µl of the MS was incubated with 5 µl phosphatidylethanolamine L-α-1 palmitoyl-2-arachidonyl [arachidon-1-14C] (360 nmol/ml, 53 µCi/mmol, New England Nuclear; 1 Ci=3.8 x 1010 Bq) and 10 µl of a solution containing one of the following substances yielding final concentrations as given in brackets: human thrombin (1 U/ml), H2O2 (0.1 and 1.0 mmol/l). For determining the basal PLA2 activity 10 µl of THC was added instead of the stimulant solution. Incubations were at 37°C for 20 or 60 min. unless otherwise stated. The reaction was terminated with 25 µl of 100% ethanol containing 8% glacial acetic acid (stop solution). Control experiments were performed either with THC only (no membranes) or with simultaneous addition of stimulant and stop solution to the MS. PLA2 activity was determined by measuring the amount of [1-14C] arachidonate released from the substrate phosphatidylethanolamine L-α-1 palmitoyl-2-arachidonyl [arachidon-1-14C]. After mixing a 20 µl fraction was applied to a 60 F254 silica gel thin-layer chromatography plate (Merck, Germany) and developed in a solvent system of ethyl acetate and glacial acetic acid 99:1 (vol/vol). Chromatography with [1-14C] arachidonic acid (Amersham) showed that free arachidonic acid migrated with the solvent while the phospholipid substrate in absence of PLA2 remained at the origin. After development of the chromatography plate the areas corresponding to free arachidonate were cut out, placed in a scintillation vial containing 8 ml of the scintillant Optiphase "High Safe II" (Pharmacia, Sweden) and the radioactivity was measured.
Statistical Analysis.
For determination of significance results were subjected to ANOVA analysis. Data are given as mean ± standard deviation (STD).

RESULTS

Time Course of IP$_3$
Addition of 1 U/ml thrombin to the cell layers led to a rapid rise in IP$_3$ formation with a maximum induction observed between 10 to 20 seconds (Figure 1A). IP$_3$ formation peaked 300 seconds following incubation with 0.1 mmol/l H$_2$O$_2$ (Figure 1B). On incubating the cells with 1.0 mmol/l H$_2$O$_2$, IP$_3$ formation was first detected 60 seconds after addition reaching its maximum after 600 seconds (164 ± 18 %; p<0.05). Similar results were observed using ATP as a stimulant (data not shown).
IP$_3$ resting levels remained unchanged when PKC was directly activated with PMA.
**Time Course of IP₂**

IP₂ formation was initiated 10 seconds following the addition of thrombin and reached its maximum at 30 seconds (Figure 1A). Thereafter, a rapid followed by a slower decrease to resting levels was observable. Stimulation of the cells with 0.1 mmol/l H₂O₂ delayed IP₂ formation peaking at 300 seconds and thereafter continuously decreased (Figure 1B). In the presence of 1.0 mmol/l H₂O₂ a statistically significant elevation in IP₂-levels (122 ± 1 %; p<0.05) was detectable 60 seconds after addition as was observable in the case of IP₃. Thirty seconds after stimulation of the cells with PMA a significant decrease in IP₂-levels was detectable compared to controls.

**Time Course of IP₁**

Stimulation of the cells with thrombin induced a continuous increase of IP₁ to maximum levels observed at between 30 to 45 seconds (Figure 1A). Thereafter a decline to resting levels occurred. A concentration of 0.1 mmol/l H₂O₂ induced a small but significant spike in IP₁ formation after 300 seconds (Figure 1B). A ten fold concentration of H₂O₂ also led to enhanced IP₁ formation after 300 seconds (122 ± 17 %; p<0.05). During PKC activation with PMA, IP₁-formation was tendentiously induced within the first 30 seconds, with resting levels remaining stable thereafter.

![Graph](image_url)

**FIG. 1B.**

Line plots showing time dependence of IP₃, IP₂ and IP₁ formation upon stimulation with 0.1 mmol/l H₂O₂ up to 600 seconds. 100% IP₃ = 1.13 pmol/10⁸ cells, 100% IP₂ = 4.16 pmol/10⁸ cells, 100% IP₁ = 5.85 pmol/10⁸ cells. Mean ± STD of six different experiments performed in triplicate. * = statistically significant (p<0.05).
Ca²⁺ signaling and PG₁₂ release

After addition of 1 U/ml thrombin, intracellular free Ca²⁺ reached its maximum after between 30 to 60 seconds (Figure 2A). Whereas 0.1 mmol/l H₂O₂ failed to elevate the levels of intracellular free Ca²⁺, (Figure 2A), a concentration of 1.0 mmol/l H₂O₂ induced a delayed rise of Ca²⁺ peaking after 300 seconds (340 ± 19 nmol/l, p<0.05). Although PMA failed to elevate IP₃, a significant rise in cytosolic Ca²⁺ was observable after 20 seconds (1860 ± 47 nmol/l; p<0.05).

Time dependent modulations of PG₁₂ release after incubation of endothelial cells with either 1 U/ml thrombin or 0.1 mmol/l H₂O₂ are depicted in Figure 2B. PG₁₂ release under the physiological stimulus thrombin started 40 seconds after onset of stimulation. In contrast, oxygen radicals induced eicosanoid release was slightly delayed. Upon stimulation with 0.1 mmol/l H₂O₂ significant levels of PG₁₂ were first detected after 60 seconds. Similar results were observed using 1.0 mmol/l H₂O₂ as a stimulant.

During the incubation period with DPBS (= control), PG₁₂-release remained unchanged (81 ± 34 pmol/10⁸ cells). PMA (1 μmol/l) failed to elevate the release of PG₁₂.

Line plots showing time dependence of cytosolic free Ca²⁺ (Fig. 2A.) and PG₁₂ synthesis (Fig. 2B.) upon stimulation with 1 U/ml thrombin or 0.1 mmol/l H₂O₂ up to 600 seconds. Mean ± STD of six different experiments performed in triplicate.

* = statistically significant (p<0.05).
PLA₂ activity in intact endothelial cells.
Incubation of the endothelial cell layers with 1 U/ml thrombin led to a strong increase in arachidonic acid liberation (increase by 322 ± 56.5 %). The incubation with the oxygen radicals generating agent H₂O₂ at concentrations of 0.1 or 1.0 mmol/l failed to induce arachidonic acid liberation (Figure 3). Pretreatment of the cell layers with either 0.1 or 1.0 mmol/l H₂O₂ and subsequent stimulation with 1 U/ml thrombin did not reduce the release of arachidonic acid into the supernatants.

PLA₂ activity in endothelial cell membranes.
Incubation of the MS with 1 U/ml thrombin resulted in greatly increased PLA₂-activity at 37° C (increase by 103 %). The incubation with the oxygen radicals generating agent H₂O₂ at concentrations of 0.1 or 1.0 mmol/l did not enhance PLA₂-activity (Figure 3).
DISCUSSION

Oxidative stress influences vascular reactivity via alterations in the synthesis and release of endothelium derived paracrine factors. In order to determine whether oxidants alter receptor-mediated Ca²⁺ signaling of the endothelial cell, we examined the effects of H₂O₂ with regard to inositol signal transduction, cytosolic free Ca²⁺ and eicosanoid formation. Furthermore, we attempted to relate our findings to the effects of a physiological stimulant (thrombin), to determine, how transduction pathways are influenced by reactive oxygen species. The agonist-stimulated increase in Ca²⁺ levels comprises two processes: influx of Ca²⁺ from the extracellular compartment, and release of Ca²⁺ from intracellular stores. It has been well established that the Ca²⁺ release from internal stores results from the generation of IP₃ from PIP₂ via G-protein dependent activation of PLC (1,13). The results obtained in this study using

Bars showing the influence of 1 U/ml thrombin or 0.1 or 1.0 mmol/l H₂O₂ on PLA₂ activity in endothelial cell membranes and intact cells. Data is given in nmol/mg protein/h or % of control, respectively. Mean ± STD of six different experiments performed in triplicate. ** = Statistically significant (p<0.001)
thrombin as a stimulant further support this mechanism, but they also show that this pathway is altered during H$_2$O$_2$ exposure. Moreover, our experiments with PMA show, that isolated increases in Ca$^{2+}$ level without formation of IP$_3$ is not sufficient to stimulate prostaglandin synthesis. Previously we could show that thrombin induced eicosanoid release is not downregulated by inhibiting IP$_3$ formation with PMA. This finding is explainable since we demonstrated in the present study that thrombin markedly enhances membrane associated PLA$_2$ activity indicating an action independent from the IP$_3$ pathway. In contrast, ATP which failed to enhance PLA$_2$ activity (data not shown) was unable to stimulate eicosanoid release after treatment of HUVEC with PMA indicating an involvement of the inositol signal transduction pathway.

H$_2$O$_2$ induces delayed increases in IP$_3$, IP$_2$ and IP$_1$—compared to the experiments with thrombin—indicating the activation of the inositol signal transduction pathway and the generation of DAG, which by itself serves as a source of arachidonic acid. Ohgushi et al. demonstrated activation of PKC by oxidatively modified LDL in a biphasic manner, whereby lower concentrations of lysophosphatidylcholine activated, higher concentrations inhibited PKC. This mechanism could account for the delayed increase of inositol phosphates under H$_2$O$_2$ stimulation (21). Significant releases of Ca$^{2+}$ from internal stores were not detectable, which is in contrast to the physiological function of IP$_3$. It appears possible that H$_2$O$_2$ inhibits the interaction of IP$_3$ with its receptor at the endoplasmic reticulum by enhancing the oxidation of glutathion (GSH) to its dimeric form (GSSG) for which an IP$_3$ receptor blockade is described (14). Windischbauer et al. have demonstrated in a recent study that GSSG is increased in human endothelial cells during hypoxia (22).

The fact, that eicosanoid synthesis is strongly enhanced by H$_2$O$_2$ independent from Ca$^{2+}$ is in good accordance with Hazen et al. who demonstrated an activation of a Ca$^{2+}$ independent 40 kDa PLA$_2$ isoenzyme in ischemic hearts (23).

An alternative explanation for eicosanoid synthesis in the presence of H$_2$O$_2$ could be based on the observation by Wijkander and Sundler (24) providing evidence that hydrolytic activity of PLA$_2$ was obtained in the absence of Ca$^{2+}$. This implies that Ca$^{2+}$ is required for interfacial association with lipid but not for catalysis. It might be speculated, that substances reducing the concentration of Ca$^{2+}$ required for maximal activity of the enzyme, could lead to eicosanoid formation in absence of significant increases in free Ca$^{2+}$. Since it was demonstrated that DAG is able to reduce the concentration of Ca$^{2+}$ required for PLA$_2$ activity the activation of this enzyme by H$_2$O$_2$ is explainable. This type of mechanism was reported by Leslie and Channon (3).

In summary, we were able to demonstrate that H$_2$O$_2$ stimulation of endothelial cells leads to significant but delayed rises in inositol phosphate formation. It might be postulated that inhibition of Ca$^{2+}$ release from internal stores is due to increases in GSSG for which inhibitory actions at the endoplasmic IP$_3$ receptor are described. Although membrane associated PLA$_2$ is not activated and cytosolic free Ca$^{2+}$ is not increased, eicosanoid formation occurs, which could involve the activation of a Ca$^{2+}$ independent, cytosolic 40 kDa PLA$_2$ isoenzyme. DAG might serve as a source of arachidonic acid and seems to sensitize a cytosolic PLA$_2$.

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