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Potentiation of Sympathetic Neurotransmission in Bovine Isolated Irides by Isoprostanes

CATHERINE A. OPERE, S. OLUBUSAYO AWE, LYDIA C. HARRIS, ANGELA M. LEDAY and SUNNY E. OHIA*

Department of Pharmaceutical and Administrative Sciences, School of Pharmacy and Allied Health Professions, Creighton University, 2500 California Plaza, Omaha, NE 68178

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Isoprostanes (IsoP) are formed by free radical catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. In the present study, we examined the effect of IsoP on norepinephrine (NE) release from the bovine isolated iris. Furthermore, we studied the role of IsoP's in hydrogen peroxide (H_2O_2)-induced enhancement of NE release from this tissue. Isolated bovine irides were prepared for studies of [^{3}H]NE release using the superfusion method. Release of [^{3}H]NE was induced via electrical field stimulation. Both 8-iso-prostaglandin E_2 (E_2-IsoP) and 8-iso-prostaglandin $F_{2\alpha}$ (F_2-IsoP) produced a concentration-related enhancement of field-stimulated [^{3}H]NE release from isolated bovine irides, an effect that was mimicked by the thromboxane (Tx) receptor agonist, U46619 and by H_2O_2. The Tx-receptor antagonist, SQ 29548 inhibited responses to E_2-IsoP (10 μM) with an IC_{50} of 370 ± 50 nM. SQ 29548 (10 μM) also blocked the enhancement of electrically-evoked [^{3}H]NE release induced by U46619 (10 μM) but not that caused by H_2O_2 (300 μM). The Tx synthetase inhibitor, carboxyheptylimidazole (10 μM) prevented the stimulatory effect of E_2-IsoP on evoked [^{3}H]NE release without affecting responses induced by H_2O_2. We conclude that IsoP's can enhance sympathetic neurotransmission in the bovine isolated iris, an effect that can be blocked by a Tx-receptor antagonist. Furthermore, endogenously produced Tx's mediate the stimulatory effect of IsoP's on NE release. However, endogenously generated IsoP's or Tx's are not involved in H_2O_2-induced potentiation of sympathetic neurotransmission.

Keywords: Isoprostanes, hydrogen peroxide, thromboxanes, iris, neurotransmission, receptors

INTRODUCTION

About 10 years ago, Morrow and co-workers[1] discovered prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-like compounds that were formed in abundance *in vivo* by free radical catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase (COX) enzyme. Because these compounds are isomeric to $PGF_{2\alpha}$ formed by COX, they were named F_2-isoprostanes (F_2-IsoP's). In addition to F_2-IsoP's, IsoP endoperoxides can undergo

*Corresponding author. Tel.: (402) 280-5927. Fax: (402) 280-5692. E-mail: seohia@creighton.edu.

rearrangement *in vivo* to form PGD_2-like (D_2-IsoP's), PGE_2-like (E_2-IsoP's) and thromboxane-like (IsoTx's) compounds.[2,3] Unlike eicosanoids derived from COX, IsoP's and IsoTx's are formed *in situ* in phospholipids and can be released preformed.[2,3] Of all IsoP's, the biological activity of F_2-IsoP's have been the most extensively studied. F_2-IsoP's are potent vasoconstrictors of renal and pulmonary arteries and there is evidence that they can induce mitogenesis in vascular smooth muscle cells and endothelin release from endothelial cells.[4] Both F_2-IsoP's and E_2-IsoP's have been shown to cause concentration-dependent decreases in coronary flow in guinea pigs, an effect that was blocked by the thromboxane A_2 (TxA_2)-receptor antagonist, SQ 29548.[5]

Hydrogen peroxide (H_2O_2), a compound capable of generating reactive oxygen species, is present in tissues of the anterior segment of the eye such as the cornea, iris-ciliary body and lens.[6–8] Evidence from our laboratory reveals that H_2O_2 can potentiate sympathetic neurotransmission in the iris-ciliary body of several mammalian species, an effect that is dependent on age of the animal.[9] Effects of H_2O_2 on evoked norepinephrine (NE) release involved generation of reactive oxygen species but was unaffected by agents that interfered with the adenylyl cyclase pathway.[10] Furthermore, the ability of H_2O_2 to enhance field-stimulated NE release was unaffected by antagonism of prejunctional α_2-adrenoceptors suggesting that these autoreceptors are not involved in the peroxide response.[11] In another study, we found that H_2O_2-induced enhancement of evoked NE release from the bovine isolated irides required the presence of trace amounts of extracellular calcium[12] and is dependent on the functional integrity of the mitochondrial calcium stores.[13] In spite of the fact that calcium has been implicated in peroxide stimulatory effects on sympathetic neurotransmission, it is unclear whether other mediators or modulators could also play a role in this response.

There is evidence that H_2O_2 can interact with the pathway leading to free radical catalyzed peroxidation of arachidonic acid independent of the COX enzyme. Salahudeen *et al.*[14] demonstrated that H_2O_2 can stimulate F_2-IsoP production in renal tubular epithelial cells, an effect that was inhibited by 21-aminosteroids. The ability of H_2O_2 to stimulate the biosynthesis of F_2-IsoP's thus confirms the role of this oxidant in initiating non-COX dependent peroxidation of arachidonic acid. In the present study, we investigated the possibility that IsoP's could mediate the effect of H_2O_2 on sympathetic neurotransmission. To test this hypothesis, we first designed studies to examine the effect of IsoP's on sympathetic neurotransmission. Secondly, we studied the effect of putative antagonists of IsoP receptors (such as the Tx-receptor antagonist, SQ 29548) on the H_2O_2 response. The aim of the present study was, therefore, to: (a) investigate the effect of IsoP's on field-stimulated [^{3}H]-norepinephrine ([^{3}H]NE) release from bovine isolated irides; and (b) to determine the contribution of IsoP's in H_2O_2-induced enhancement of sympathetic neurotransmission in this tissue. Parts of the result presented in this paper have been communicated as an abstract.[15]

MATERIALS AND METHODS

Studies were performed with cow eyeballs obtained from slaughterhouses in the Omaha metropolitan area (Greater Omaha Packing Co. Inc., and J.F. O'Neill Packing Co.). Eyeballs from freshly sacrificed cows were transported to the laboratory in an ice bucket. The anterior segment of each eye was removed and placed immediately in oxygenated Krebs solution and irides were dissected free of the lens, lens-capsule, sclera and adherent vitreous. Hemi-irides (5 mm wide, 25 mm long) were then prepared for *in vitro* superfusion assays.

Studies on [^{3}H]-Norepinephrine Release

Tissues were prepared for analysis of evoked [^{3}H]-norepinephrine ([^{3}H]NE) release as described

previously.[9,16] Briefly, isolated bovine irides were mounted between nylon mesh cloth (200 μm pore size) and incubated for 60 min at 37 °C in a bicarbonate-buffered, carbogen-gassed Krebs buffer solution containing 2.5 μCi/ml L-[^{3}H]NE (New England Nuclear, Boston, MA; 40.8 Ci/mmol) with or without flurbiprofen (3 μM). The Krebs solution had the following composition (mM): NaCl, 112; KCl, 4.7; $CaCl_2$, 2.2; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; ascorbic acid 0.1; and glucose, 14 (pH 7.4). After incubation, tissues were rinsed (three 5 min washes) in warm Krebs buffer solution to remove excess radioactivity and then transferred to individual, temperature-controlled Plexiglass superfusion chambers. Irides were superfused at 2 ml/min with warm oxygenated Krebs buffer solution containing the neuronal uptake inhibitor, desipramine (1 μM). Fractions of the superfusate were collected at 4 min intervals and 4 ml aliquots of each fraction were combined with 12 ml of aqueous scintillation cocktail (Ecolume, ICN Radiochemicals, CA) and analyzed for radioactivity by liquid scintillation spectrometry.

After an initial 60 min of superfusion to establish a stable baseline of spontaneous tritium efflux, release of [^{3}H]NE was elicited by consecutive trains of 300 d.c. pulses (5 Hz, 2 msec pulse duration, 50 V/cm interelectrode distance, 60s) delivered to the platinum chamber electrodes using a Grass S48 stimulator (Grass Instruments, Quincy, MA). Stimulation-evoked release and overflow of tritium was estimated by subtraction of the extrapolated basal tritium efflux from total tritium released during the 20 min period after the onset of stimulation. Basal (unstimulated) tritium efflux was assumed to decline linearly between pre- and post-stimulation fractions. Based on chromatographic analysis of the radioactive release products in bovine irides, stimulation evoked tritium efflux was designated as [^{3}H]NE release.[16]

All tissues received two stimulations (S_1 and S_2) 30 min apart. To examine the effect of IsoP's, H_2O_2 and other compounds on [^{3}H]NE release, test agents were applied 8 min before and during S_2. To determine the effect of antagonists on IsoP and H_2O_2-induced enhancement of field-stimulated [^{3}H]NE release from mammalian irides, these agents were present in the buffer solution 20 min before S_1 and also during S_2. Stimulation-evoked release of [^{3}H]NE during S_1 and S_2 was determined graphically, and the ratio of the two peaks (S_2/S_1) was calculated and compared to untreated control preparations. The pattern and total amount of basal and evoked [^{3}H]-NE efflux from isolated, superfused tissues can vary remarkably. However, the ratio of evoked [^{3}H]-NE overflow between the first (S_1) and second (S_2) stimulation period is near unity for control experiments but varies under test conditions.

Data Analysis

Results obtained were expressed as percentage increase in [^{3}H]NE release or as absolute S_2/S_1 ratios. Data from different experiments (control and test) were pooled and then subjected to statistical analysis. Except where indicated otherwise, values given are arithmetic means ± SEM. Significance of differences between S_2/S_1 values obtained in control and agent-treated preparations were evaluated using analysis of variance (ANOVA) followed by Dunnett's test (GraphPad Software, San Diego, CA). Differences with P values < 0.05 were accepted as statistically significant.

Drugs and Chemicals

1(7-carboxyheptyl)imidazole hydrochloride was obtained from Tocris Cookson Inc. SQ 29548 and flurbiprofen were purchased from R.B.I. Chemical Company, U46619, 8-iso-prostaglandin E_2 and 8-iso-prostaglandin $F_{2\alpha}$ were obtained from Cayman chemical company while hydrogen peroxide was purchased from Sigma Chemical (St. Louis, MO). L-[^{3}H]-norepinephrine (40–80 Ci/mmol) was purchased from Dupont NEN, Boston, MA.

RESULTS

Electrical field stimulation of irides preloaded with [^{3}H]NE caused an overflow of tritium efflux over basal levels. In the first series of experiments, we compared the effect of H_2O_2 on sympathetic neurotransmission with those of E_2-IsoP and F_2-IsoP. As illustrated in Figure 1, application of E_2-IsoP (1 μM), F_2-IsoP (30 μM) and H_2O_2 (300 μM) 8 min before the second period of stimulation (S_2) potentiated electrically-evoked [^{3}H]NE release without affecting basal tritium efflux. Furthermore, both E_2-IsoP and F_2-IsoP caused a concentration-dependent enhancement of evoked [^{3}H]NE overflow (Figure 2). At concentrations less than 10 μM, F_2-IsoP caused slight inhibitions of evoked [^{3}H]NE release. Since most pharmacological actions of IsoP's can be mimicked by the Tx-receptor agonist, U46619,[17,18] we also examined the effect of this prostanoid on field-stimulated [^{3}H]NE release. As observed with the IsoP's, U46619 also caused a concentration-related enhancement of evoked [^{3}H]NE overflow (Figure 2).

Because of the susceptibility of IsoP responses to blockade by SQ 29548,[5,17] we examined the effect of this Tx-receptor antagonist on enhancements of field-stimulated [^{3}H]NE release induced by E_2-IsoP, U46619 and H_2O_2. In the concentration range, 100 nM to 10 μM, SQ 29548 antagonized responses to E_2-IsoP (10 μM) with an IC_{50} of 370 ± 50 nM ($n = 4$). SQ 29548 (10 μM)

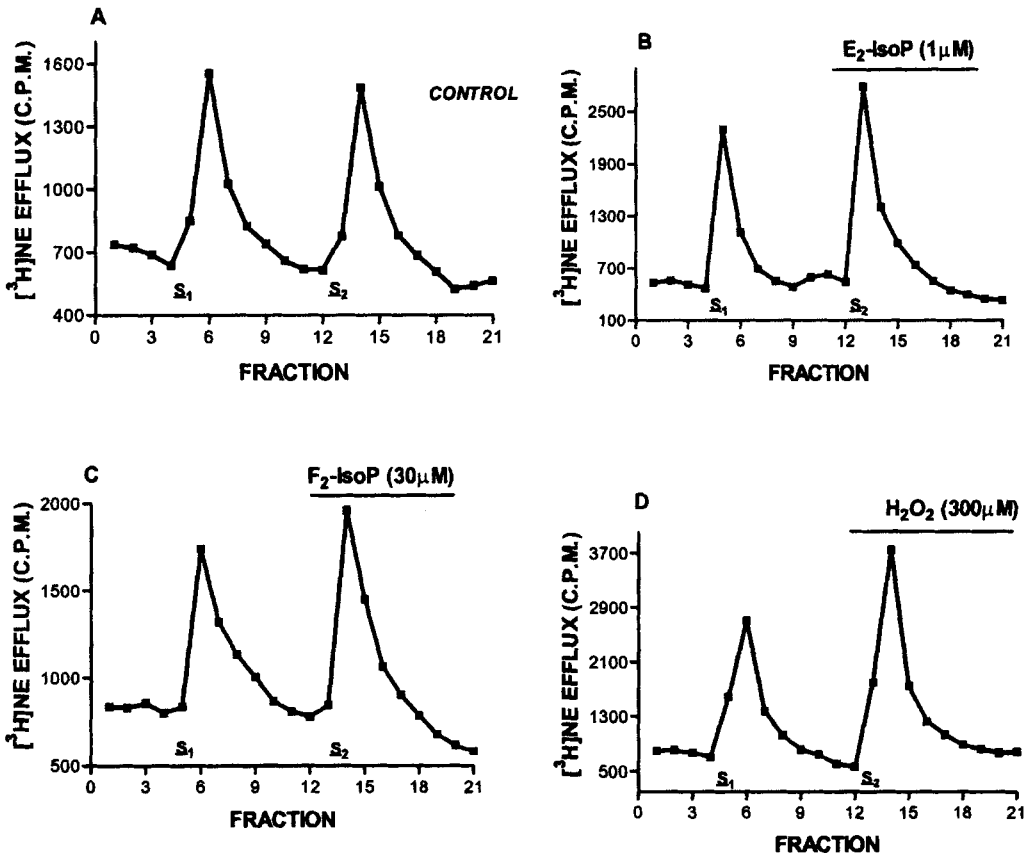

FIGURE 1 Effect of 8-iso-prostaglandin E_2 (E_2-IsoP), 8-iso-$PGF_{2\alpha}$ (F_2-IsoP) and hydrogen peroxide (H_2O_2) on field-stimulated [^{3}H]-norepinephrine ([^{3}H]NE) release from isolated, superfused bovine irides: control (panel A) and in the presence of E_2-IsoP (1 μM; panel B), F_2-IsoP (30 μM; panel C) and H_2O_2 (300 μM; panel D). IsoP's and H_2O_2 were applied 8 min before and during S_2. Trains of field stimulation (5 Hz, 2 msec p.d., supramaximal voltage, 60 s) were applied at fraction 5 (S_1) and fraction 13 (S_2).

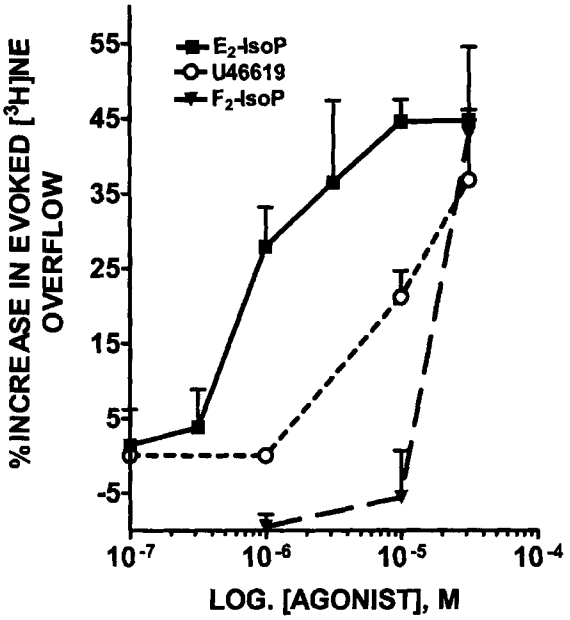

FIGURE 2 Concentration-related enhancement of field-stimulated [^{3}H]-norepinephrine ([^{3}H]NE) release from isolated, superfused bovine irides by 8-iso-prostaglandin E_2 (E_2-IsoP), 8-iso-prostaglandin $F_{2\alpha}$ (F_2-IsoP) and the thromboxane receptor agonist, U46619. Vertical bars represent the mean ± S.E.M. of four to eight determinations.

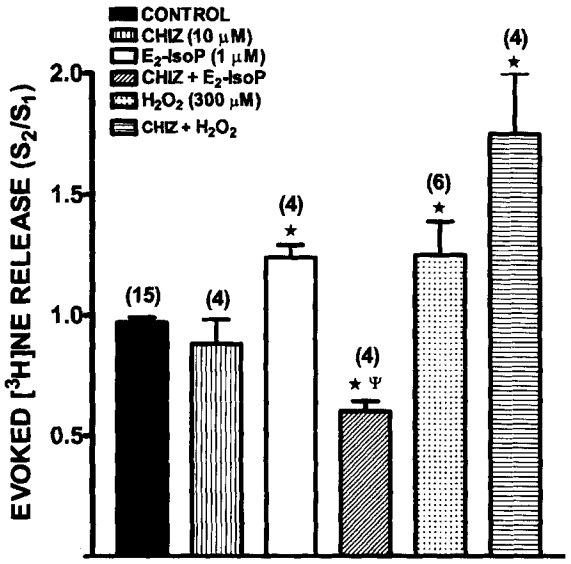

FIGURE 3 Effect of thromboxane synthetase inhibitor, carboxyheptylimidazole (CHIZ) on 8-iso-prostaglandin E_2 (E_2-IsoP) and hydrogen peroxide (H_2O_2)-induced potentiation of field-stimulated [^{3}H]-norepinephrine ([^{3}H]NE) release from isolated, superfused bovine irides: control and in the presence of CHIZ (10 μM), E_2-IsoP (1 μM), H_2O_2 (300 μM), CHIZ plus E_2-IsoP and CHIZ plus H_2O_2. Vertical bars represent mean ± S.E.M. Number of observations is in parentheses. *$P < .001$, significantly different from untreated control. $^{\Psi}P < .001$, significantly different from E_2-IsoP alone.

also blocked the enhancement of electrically-evoked [^{3}H]NE overflow induced by U46619 (10 μM) but not that caused by H_2O_2 (300 μM) (Table I).

We next considered the possibility that endogenous Tx's could mediate the enhancement of field-stimulated [^{3}H]NE release induced by E_2-IsoP and H_2O_2. As illustrated in Figure 3, pretreatment of tissues with the Tx synthetase inhibitor, carboxyheptylimidazole (CHIZ, 10 μM) prevented and even reversed the stimulatory effect of E_2-IsoP (1 μM) on evoked [^{3}H]NE release. However, the enhancement of evoked [^{3}H]NE overflow caused by H_2O_2 (300 μM) was not prevented by CHIZ (10 μM) (Figure 3). The slight enhancement of the H_2O_2 response observed in the presence of CHIZ was not significantly different ($p = 0.08$) from the effect caused by H_2O_2 alone.

TABLE I Effect of SQ 29548 on U46619 and H_2O_2-induced potentiation of field-stimulated [^{3}H]NE release in bovine isolated iris

Experiment	S_2 / S_1
Control	0.97 ± 0.02 (15)
SQ 29548 (10 μM)*	0.96 ± 0.08 (4)
U46619 (10 μM)*	1.18 ± 0.03 (4)†
H_2O_2 (300 μM)*	1.34 ± 0.10 (6)†
SQ 29548 + U46619**	0.99 ± 0.08 (4)
SQ 29548 + H_2O_2**	1.33 ± 0.11 (4)†

Values given are mean ± S.E.M. Number of observations is in parentheses; * SQ 29548, U46619 and H_2O_2 were added 8 min before S_2; ** SQ 29548 was added 20 min before tissues were stimulated at S_1 and was also present during S_2; † $P < 0.001$, significantly different from control.

DISCUSSION

Since the discovery of IsoP's by Morrow *et al.*[1] evidence from biomedical literature supports the fact that these unique products of lipid peroxidation can provide a reliable measure of oxidant

injury both *in vivo* and *in vitro*.[18–21] In addition to their role as markers of oxidative stress, IsoP's have been shown to exert pharmacological effects on the cardiovascular system.[22,23] Oxidative stress induced by H_2O_2 has been shown to enhance field-stimulated [^{3}H]NE release from isolated, superfused mammalian irides.[9] Although second messengers such as calcium have been implicated in the stimulatory effect of H_2O_2 on sympathetic neurotransmission in bovine isolated irides,[12,13] it is unclear whether other mediators or modulators could be involved in this response. Based on an earlier report that H_2O_2 can stimulate F_2-IsoP biosynthesis in the kidneys,[14] the present study was designed to investigate the role of IsoP's in peroxide-induced enhancement of sympathetic neurotransmission in the bovine iris. Conceivably, as mediators of the H_2O_2 response, IsoP's could have a direct effect on field-stimulated [^{3}H]NE release. Both E_2-IsoP and F_2-IsoP caused a concentration-dependent enhancement of field-stimulated [^{3}H]NE release from isolated bovine irides. We observed that F_2-IsoP caused a similar enhancement of electrically-evoked [^{3}H]NE release from isolated human iris-ciliary bodies whereas E_2-IsoP elicited an inhibitory response in this tissue.[24] Taken together, these results show that there is species difference in the response of sympathetic nerves in the bovine and human anterior uvea to E_2-IsoP but not to F_2-IsoP.

Because of the similarity between the pharmacological effects of IsoP's with those of Tx agonists,[5,17] we compared the effect elicited by IsoP's with those caused by U46619 on sympathetic neurotransmission in the bovine isolated iris. Indeed, Tx's have also been shown to enhance sympathetic neurotransmission in tissues such as the rabbit vas deferens,[25] portal veins[26] and mesenteric arteries.[27] U46619 also caused a concentration-related potentiation of evoked [^{3}H]NE release from the bovine isolated iris suggesting that Tx's may also regulate adrenergic neurosecretion in this tissue.

It is interesting to note that both non-COX and COX-derived metabolites of arachidonic acid and a free radical intermediate (H_2O_2) caused enhancements of electrically-evoked [^{3}H]NE release from the bovine iris. At concentrations that induced a 20% increase in field-stimulated [^{3}H]NE overflow, the rank order of potency for IsoP's and the Tx-receptor agonist was: E_2-IsoP > U46619 > F_2-IsoP. Although F_2-IsoP was the least potent of all agents tested, it caused slight inhibitory responses at concentrations less than 10 μM. These inhibitory responses were absent in tissues pretreated with the COX-inhibitor, flubiprofen indicating a possible effect of low concentrations of F_2-IsoP on prostanoid formation. The observed similarity in the ability of IsoP's, U46619 and H_2O_2 to enhance electrically-evoked [^{3}H]NE would suggest a common pathway for their effects on neurotransmission.

A prominent feature of the pharmacological action of IsoP's in vascular smooth muscle is their susceptibility to blockade by SQ 29548, a TxA_2-receptor antagonist.[5,17] In the present study, SQ 29548 antagonized the enhancement of field-stimulated [^{3}H]NE release induced by E_2-IsoP with an IC_{50} in the same range as has been reported for blockade of F_2-IsoP induced responses by this antagonist in porcine coronary arteries.[28] We also observed that the enhancement of electrically-evoked [^{3}H]NE release induced by U46619 was blocked by SQ 29548. Taken together, these results indicate that Tx-receptors may mediate the enhancement of field-stimulated [^{3}H]NE release caused by both E_2-IsoP and U46619 in the bovine iris.

We next examined the effect of Tx-receptor blockade on H_2O_2-induced potentiation of NE release from bovine isolated irides. SQ 29548 did not block H_2O_2-induced potentiation of evoked [^{3}H]NE overflow suggesting that Tx-receptors are not involved in the stimulatory effect of H_2O_2 on sympathetic neurotransmission in this tissue.

In vitro, H_2O_2 has been shown to stimulate Tx production in rat and guinea pig lungs, an effect

that can be blocked by the Tx synthetase inhibitor, CHIZ.[29,30] Although Tx-receptors are not directly involved in the enhancement of evoked [^{3}H]NE release caused by H_2O_2, it is feasible that metabolites from the Tx synthetase pathway could still contribute to the peroxide response. CHIZ abolished the stimulatory effect of E_2-IsoP on electrically-evoked [^{3}H]NE release but it had no significant action ($P > 0.05$) on the H_2O_2 response. These results indicate that endogenous Tx's are involved in the E_2-IsoP response. However, endogenous Tx's do not mediate the excitatory effects of H_2O_2 on sympathetic neurotransmission in the bovine isolated iris.

We conclude that both E_2-IsoP and F_2-IsoP can potentiate field-stimulated [^{3}H]NE release from bovine isolated irides, an effect that can be mimicked by the Tx agonist, U46619 and blocked by a Tx-receptor antagonist. Furthermore, endogenously generated Tx's may play a role in the enhancement of sympathetic neurotransmission induced by IsoP's. On the other hand, the stimulatory effect of H_2O_2 on evoked NE release from bovine irides is neither mediated by endogenously produced IsoP's or Tx's nor by Tx-receptors.

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