Regulation of $K_{Ca}2.3$ and endothelium-dependent hyperpolarization (EDH) in the rat middle cerebral artery: the role of lipoxygenase metabolites and isoprostanes

Background and purpose: In rat middle cerebral arteries, endothelium-dependent hyperpolarization (EDH) is mediated by activation of calcium-activated potassium (K_{Ca}) channels specifically $K_{Ca}2.3$ and $K_{Ca}3.1$. Lipoxygenase (LOX) products function as endothelium-derived hyperpolarizing factors (EDHFs) in rabbit arteries by stimulating $K_{Ca}2.3$. We investigated if LOX products contribute to EDH in rat cerebral arteries. **Methods:** Arachidonic acid (AA) metabolites produced in middle cerebral arteries were measured using HPLC and LC/MS. Vascular tension and membrane potential responses to SLIGRL were simultaneously recorded using wire myography and intracellular microelectrodes. **Results:** SLIGRL, an agonist at PAR2 receptors, caused EDH that was inhibited by a combination of $K_{Ca}2.3$ and $K_{Ca}3.1$ blockade. LOX- inhibition reduced EDH. Soluble epoxide hydrolase (sEH) inhibition enhanced the $K_{Ca}2.3$ component of EDH. Following NO synthase (NOS) inhibition, the $K_{Ca}2.3$ component of EDH was absent. Using HPLC, middle cerebral arteries metabolized 14C-AA to 15- and 12-LOX products under control conditions. With NOS inhibition, there was little change in LOX metabolites, but increased F-type isoprostanes. 8-iso-PGF$_{2alpha}$ inhibited the $K_{Ca}2.3$ component of EDH. **Conclusions:** LOX metabolites mediate EDH in rat middle cerebral arteries. Inhibition of sEH increases the $K_{Ca}2.3$ component of EDH. Following NOS inhibition, loss of $K_{Ca}2.3$ function is independent of changes in LOX production or sEH inhibition but due to increased isoprostane production and subsequent stimulation of TP receptors. These findings have important implications in diseases associated with loss of NO signalling such as stroke; where inhibition of sEH and/or isoprostane formation may of benefit.

1 **Regulation of $K_{Ca}2.3$ and endothelium-dependent hyperpolarization (EDH) in the rat**

2 **middle cerebral artery: the role of lipoxygenase metabolites and isoprostanes.**

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15 **Running title: EDH, LOX and isoprostanes**

16   **Background and purpose**. In rat middle cerebral arteries, endothelium-dependent

17   hyperpolarization (EDH) is mediated by activation of calcium-activated potassium (K_{Ca}) channels

18   specifically $K_{Ca}2.3$ and $K_{Ca}3.1$. Lipoxygenase (LOX) products function as endothelium-derived

19   hyperpolarizing factors (EDHFs) in rabbit arteries by stimulating $K_{Ca}2.3$. We investigated if LOX

20   products contribute to EDH in rat cerebral arteries.

21   **Methods** Arachidonic acid (AA) metabolites produced in middle cerebral arteries were measured

22   using HPLC and LC/MS. Vascular tension and membrane potential responses to SLIGRL were

23   simultaneously recorded using wire myography and intracellular microelectrodes.

24   **Results** SLIGRL, an agonist at PAR2 receptors, caused EDH that was inhibited by a combination

25   of $K_{Ca}2.3$ and $K_{Ca}3.1$ blockade. Non-selective LOX- inhibition reduced EDH, whereas inhibition

26   of 12-LOX had no effect. Soluble epoxide hydrolase (sEH) inhibition enhanced the $K_{Ca}2.3$

27   component of EDH. Following NO synthase (NOS) inhibition, the $K_{Ca}2.3$ component of EDH

28   was absent. Using HPLC, middle cerebral arteries metabolized ^{14}C-AA to 15- and 12-LOX

29   products under control conditions. With NOS inhibition, there was little change in LOX

30   metabolites, but increased F-type isoprostanes. 8-iso-$PGF_{2\alpha}$ inhibited the $K_{Ca}2.3$ component of

31   EDH.

32   **Conclusions** LOX metabolites mediate EDH in rat middle cerebral arteries. Inhibition of sEH

33   increases the $K_{Ca}2.3$ component of EDH. Following NOS inhibition, loss of $K_{Ca}2.3$ function is

34   independent of changes in LOX production or sEH inhibition but due to increased isoprostane

35   production and subsequent stimulation of TP receptors. These findings have important

36   implications in diseases associated with loss of NO signaling such as stroke; where inhibition of

37   sEH and/or isoprostane formation may of benefit.

39   **Key words:** $K_{Ca}2.3$, EDH (endothelium dependent hyperpolarization), EDHF (endothelium

40   dependant hyperpolarizing factor), Isoprostane, Lipoxygenase, HEETA, NO, cerebral arteries.

42 **Introduction**

43 Endothelium dependent hyperpolarization (EDH) is an extremely important vasodilator pathway,

44 particularly in resistance size arteries, and has a major role in regulation of blood pressure; as

45 such the EDH pathway has become intensively studied and targeting this pathway could result in

46 new classes of antihypertensive and possibly antithrombotic therapies . The exact nature of EDH

47 is still unresolved but involves several different mechanisms that include release of diffusible

48 factors (endothelium derived hyperpolarizing factors; EDHFs), direct myo-endothelial cell

49 coupling via gap junctions and increases in extracellular potassium concentration in the myo-

50 endothelial space   that all result in smooth muscle cell hyperpolarization. The relative

51 contribution of each mechanism varies between vascular beds and species .

52

53 Despite heterogeneity in the pathway, a feature of EDH responses in most vascular beds is the

54 critical role of endothelial cell calcium-activated potassium channels (K_{Ca}).   Blockade of both

55 $K_{Ca}2.3$ (SK_{Ca}) and $K_{Ca}3.1$ (IK_{Ca}) is required to fully inhibit EDH responses . In contrast, in the rat

56 middle cerebral artery (MCA), inhibition of $K_{Ca}3.1$ channels alone is sufficient to fully block the

57 EDH response obtained but only if NO synthase (NOS) is inhibited . $K_{Ca}2.3$ channels are

58 expressed in the endothelium of MCA and contribute to EDH of smooth muscle cells (SMC) in

59 vessels that are able to synthesize NO . $K_{Ca}2.3$ channel function is lost following inhibition of

60 NOS , but can be restored upon inhibition of the thromboxane prostanoid (TP) receptor and

61 associated subsequent Rho kinase mediated signalling .

62

63 In the MCA, EDH responses are also attenuated by inhibition of PLA_2 suggesting a role for a

64 metabolite of arachidonic acid (AA). A similar inhibition of EDH by PLA_2 inhibitors has also

65 been reported in peripheral arterial beds including mesenteric and coronary arteries . AA

66 metabolism is complex and produces a large number of biologically active metabolites that affect

67 vascular tone . AA metabolites produced by the cyclooxygenase (COX), epoxygenase and

68 lipoxygenase (LOX) pathways modulate K_{Ca}. The 12- and 15-LOX pathways of AA metabolism

69 have been implicated in the EDH response . Metabolites of 15-LOX, 15-hydroxy-11,12-

70 epoxyeicosatrienoic acid (HEETA) and its soluble epoxide hydrolase (sEH) metabolite, 11,12,15-

71 trihydroxyeicosatrienoic acid (THETA), have been characterized as EDHFs in rabbit arteries

72 where they stimulate $K_{Ca}2.3$ channels . Rats express a LOX that has both 12- and 15-LOX

73 activity . Therefore, it is possible that LOX metabolites acting on $K_{Ca}2.3$ could also contribute

74 EDH in the rat MCA.

75

76 NO can potentially interact with the synthesis of many AA metabolites by binding to the heme

77 group of these enzymes . Therefore, TP receptor-mediated inhibition of $K_{Ca}2.3$ following

78 inhibition of NOS may be due to altered synthesis of AA metabolites such as thromboxane A_2

79 (TxA_2) or isoprostanes that stimulate the TP receptor. Alternately following inhibition of NOS,

80 there may be a change in production of AA metabolites that normally stimulate $K_{Ca}2.3$.

81

82 As changes in AA metabolism following inhibition of NOS could potentially affect $K_{Ca}2.3$

83 function in the MCA, these studies were designed to identify the AA metabolites and determine if

84 these metabolites contribute to EDH in the MCA. Second, we investigated the effect of NOS

85 inhibition on AA metabolism and if any change in metabolite production contributes to the loss of

86 $K_{Ca}2.3$ function.

87

88 **Materials and Methods**

89 Male Wistar or Sprague Dawley rats (200-300g, circa 8-10 weeks old) were humanely killed in

90 accordance with local ethics committee guidelines. The brain was removed and immediately

91   placed in ice-cold Krebs' solution. Segments of the middle cerebral artery (~2mm long) were

92   dissected and stored in ice-cold Krebs' for use (usually within 30 min but if first vessel failed to

93   meet the exclusion criteria detailed below a second vessel would be mounted this was never >4

94   h), with similar size vessels used in all experimental groups.

95   *Simultaneous measurement of membrane potential and tension*

96   Smooth muscle membrane potential was measured simultaneously with vasomotor responses

97   using isometric recording technique. Segments of MCA (internal diameter ~150 μm) were

98   mounted in a Mulvany-Halpern myograph (model 400A, Danish Myotechnology) in Krebs'

99   solution containing (mM): NaCl, 118.0, $NaCO_3$, 24; KCl, 3.6; $MgSO_4 \cdot 7H_2O$, 1.2; glucose, 11.0;

100    $CaCl_2$, 2.5; gassed with 21% O_2, 5% CO_2, balance N_2 and maintained at 37°C. After equilibration

101   for 20 min, vessels were tensioned to 1-1.5mN (approximates wall tension at 60 mmHg). Smooth

102   muscle tension was recorded with an isometric pressure transducer and Powerlab software (ADI,

103   Australia). Vessel viability was assessed by adding exogenous K^+ (15-55 mM, total K^+

104   concentration); only vessels developing tension of ≥3 mN were used. Endothelial cell viability

105   was assessed by the ability of an agonist of protease activated receptor 2, SLIGRL (serine,

106   leucine, isoleucine, glycine, arginine, leucine; 20 μM) to relax spontaneous tone and to

107   hyperpolarize the smooth muscle cell membrane by >15 mV. SLIGRL was chosen to stimulate

108   the endothelium as more commonly used agonists such as ACh are unable to evoke an EDH

109   response in the MCA and purinergic agonists that can stimulate EDH responses evoke

110   constriction via smooth muscle cell action.

111   All endothelium-dependent responses to SLIGRL (20 μM) were obtained in resting vessels with

112   no vasoconstrictor stimulus other than spontaneous tone unless stated otherwise. EDH of smooth

113   muscle cells was assessed in the presence of the K_{Ca} channel blockers, apamin (K_{Ca}2.3, 50 nM),

114   TRAM-34 ($K_{Ca}3.1$, 1μM) and iberiotoxin ($K_{Ca}1.1$, 100 nM). The effect of K_{Ca} blockers on EDH

115   responses was also assessed after addition of three structurally distinct non-selective inhibitors of

116   LOX (ebselen, nordihydroguaiaretic acid (NGDA), both 1 μM, and PD146176, 5 μM), soluble

117   epoxide hydrolase (sEH) inhibitor tAUCB (10 μM) and isoprostane isoPGF$_{2\alpha}$ (1 μM). Isolated

118   EDH responses were obtained in the presence of the NO synthase inhibitor L-NAME (100 μM).

119   In these experiments, the effect of the K_{Ca} channel blockers was assessed on EDH responses

120   induced by SLIGRL (20 μM) in the presence of tAUCB or tempol (100 μM). Papaverine (150

121   μM) was added at the end of each experiment to assess overall tone and all relaxations are

122   expressed as a percentage of this maximal relaxation. All drugs were allowed to equilibrate for at

123   least 20 min before EDH responses were stimulated. In most experiments, smooth muscle

124   membrane potential (E_m) and tension were measured simultaneously as previously described,

125   using glass microelectrodes (filled with 2 M KCl; tip resistance, 80-120 MΩ) to measure E_m . In

126   experiments where vessels were able to synthesise NO we only reported EDH of the smooth

127   muscle cell layer as NO was able to evoke relaxation even if EDH was blocked.

128   *Measurement of metabolites of ^{14}C-labelled AA*

129   Rat cerebral arteries were dissected, cleaned, cut into 2-3 mm rings, and incubated at 37°C with

130   indomethacin (10 μM) and vehicle or indomethacin and L-NAME (100 μM) in 2 ml of N-2-

131   hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (mM): 10 HEPES, 150 NaCl, 5

132   KCl, 2 CaCl$_2$, 1 MgCl$_2$, 6 glucose, pH 7.4. After 10 min, [^{14}C]-AA (0.5 μCi, 10^{-7} M) was added,

133   incubation was continued for 5 min, and then A23187 (10 μM) was added. After 15 min, the

134   reaction was stopped with ethanol (15% final concentration). The incubation buffer was removed

135   and extracted using Bond Elute octadecylsilyl columns as previously described . The extracts of

136   the media were analyzed by reverse phase high-pressure liquid chromatography (HPLC) using a

137   Nucleosil C-18 (5μ, 4.6 x 250 mm) column . Solvent system 1 was used to resolve lipoxygenase

138   and epoxygenase metabolites and consisted of a 40 min linear gradient (flow rate=1 ml/min) from

139   50% solvent B (acetonitrile with 0.1% glacial acetic acid) in solvent A (deionized water) to 100%

140   solvent B. Column effluent was collected in 0.2 ml fractions, and the radioactivity was

141   determined. In a separate series of incubations, arterial rings were incubated with vehicle or L-

142   NAME (100 μM) as described above. Following extraction of the media, prostaglandins and

143   isoprostanes were resolved using a Nucleosil C-18 column using solvent system 2. This solvent

144   system consisted of an 40 min isocratic elution with 31% solvent B (acetonitrile) in solvent A

145   (water containing 0.025 M phosphoric acid) followed by a 20 min linear gradient to 100% B and

146   a 10 min elution with 100% B. The column eluate was collected in 0.5 ml fractions and

147   radioactivity determined. Total radioactivity from a sample was normalized to tissue weight.

148   The extracts were analyzed using liquid chromatography-mass spectrometry (LC/MS) with an

149   Agilent Model 1100SL mass selective detector and electrospray ionization . Metabolites were

150   resolved using a Kromasil C-18 (5 μm, 2 x 250 mm) column. Solvent system 3 consisted of a 10

151   min linear gradient from 15% solvent B (acetonitrile) in solvent A (water containing 0.005%

152   glacial acetic acid) to 60% solvent B in A, a 10 min linear gradient to 80% solvent B in A

153   followed by 5 min gradient to 100% B. The flow rate was 0.2 ml/min. The drying gas flow was

154   12 l/min. The nebulizer pressure was 35 psig, vaporizer temperature was 325°C and desolvation

155   temperature 350°C. The capillary voltage was 3 kV and fragmentor voltage was 120 V.

156   Detection was in the negative ion mode. For isoprostanes, the eluate was monitored at m/z 351

157   (M-H) for PGE_2-type isoprostanes and m/z 353 for PGF_2-type isoprostanes. Comparisons were

158   made with the elution times of known isoprostane standards.

159   *Data analysis and statistical procedures*

160   Results are expressed as the mean ± s.e.m. of *n* animals. Tension values are given in mN (always

161   per 2 mm segment) and E_m as mV. Vasodilatation is expressed as percentage reduction of

162   the total vascular tone (spontaneous tone plus vasoconstrictor response), quantified by

163   relaxation with papaverine (150 µM). Graphs were drawn and comparisons made using

164   one-way ANOVA with Tukeys' post-test or Students' t-test, as appropriate (Prism,

165   GraphPad). $P \leq 0.05$ was considered significant.

166

167   *Drugs, chemicals, reagents and other materials*

168   Exogenous K^+ was added as an isotonic physiological salt solution in which all the NaCl was

169   replaced with an equivalent amount of KCl. Concentrations of K^+ used are expressed as final bath

170   concentration. Ebselen (2-Phenyl-1,2-benzisoselenazol-3(2H)-one), L-NAME (N^G-nitro-L-

171   arginine methyl ester), Nordihydroguaiaretic acid (NDGA), papaverine HCl, PD 146176 (6,11-

172   Dihydro[1]benzothiopyrano[4,3-b]indole), tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-

173   oxyl), TRAM-34 (1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole) and U46619 (9,11-

174   Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) were all obtained from Sigma (Poole, U.K.).

175   Apamin and iberotoxin, from Latoxan (Valence, France). SLIGRL-NH_2 (serine, leucine,

176   isoleucine, glycine, arginine, leucine) from Auspep (Parkville, Australia). 8-iso $PGF_{2\alpha}$ from

177   Cayman-Europe (Tallinn, Estonia). tAUCB (*trans*-4-[4-(3-Adamantan-1-yl-ureido)-

178   cyclohexyloxy]-benzoic acid) was a generous gift from Professor Bruce D. Hammock

179   (University of California, Davis). All stock solutions (100 mM) were prepared in

180   dimethylsulfoxide (DMSO) except L-NAME, apamin, iberiotoxin, papaverine and SLIGRL that

181   were dissolved in 0.9% NaCl and tempol which was dissolved in ultrapure water. Vehicle

182   controls were performed when necessary.

183

184   *Abbreviations*

185   12-HETE, 12-Hydroxyeicosatetraenoic acid; AA, arachidonic acid; COX, cyclooxygenase;

186   CYP450, cytochrome P450 epoxygenases; EDH, endothelium dependent hyperpolarization;

187   EDHF, endothelium derived hyperpolarizing factor; EETs, epoxyeicosatrienoic acids; HEETA,

188   15-hydroxy-11,12-epoxyeicosatrienoic acid; $K_{Ca}1.1$, large conductance calcium-activated

189   potassium channel (BK_{Ca}); $K_{Ca}2.3$, small conductance calcium-activated potassium channel

190   (SK_{Ca}); $K_{Ca}3.1$; intermediate conductance calcium-activated potassium channel (IK_{Ca}); LOX,

191   lipoxygenase; NOS, NO synthase; sEH, soluble epoxide hydrolase; SLIGRL, serine, leucine,

192   isoleucine, glycine, arginine, leucine; THETA, 11,12,15-trihydroxyeicosatrienoic acid; TxA_2,

193   thromboxane A_2; TP, thromboxane prostanoid receptor.

194

195
196   **Results**

197   *Effect of LOX and sEH inhibition on EDH.*

198   In our previous studies, control arteries that had not been treated with a NOS inhibitor

199   demonstrate endothelium-dependent hyperpolarization (EDH) that has components from $K_{Ca}2.3$,

200    $K_{Ca}3.1$ and $K_{Ca}1.1$ channels . A similar pattern was seen in this study. Control EDH was

201   significantly inhibited only by the combination of the KCa3.1 inhibitor TRAM-34 and the

202   KCa2.3 inhibitor apamin; TRAM-34 alone had no significant inhibition alone (Figure 1B);

203   apamin alone was not assessed in this study but our previous studies demonstrate alone it does

204   not have a significant effect on EDH under these conditions (McNeish & Garland 2007; McNeish

205   et al. 2006). The residual EDH was blocked by the inhibitor of $K_{Ca}1.1$ (BK_{Ca}), iberiotoxin.

206 SLIGRL (20µM) evoked EDH (30.0±5.5 mV, n=4) that was significantly reduced by the LOX

207 inhibitor nordihydroguaiaretic (NDGA, 1µM; 10.7±2.3mV; n=4, P<0.05; Figure 1A, C). This

208 hyperpolarization was further reduced by TRAM-34 (4.3±2.4mV, n=5) but not by subsequent

209 additions of apamin and iberiotoxin (Figure 1B). NDGA also abolished spontaneous tone

210 (1.9±0.6 Vs 0.2±0.2 mN, pre- and post-NDGA, respectively, n=4, P<0.05). This relaxation was

211 not associated with significant hyperpolarization (-44.6±3.0 vs 51.9±4.3mV, pre- and post-

212 NDGA, respectively, n=4). A structurally distinct blocker of LOX, ebselen (10µM), had similar

213 effects. Ebselen significantly inhibited EDH (28.8±2.2mV, vs. 15.6±2.9mV, pre- and post-

214 ebselen, respectively; P<0.05, n=6; Figure 1C). Hyperpolarization that was resistant to ebselen

215 was not significantly reduced by TRAM-34 (Figure 1C) or further addition of apamin but the

216 subsequent addition of iberiotoxin reduced hyperpolarization further (figure 1D). Ebselen also

217 reduced spontaneous tone (0.6 ± 0.0 mN vs 0.3 ± 0.1 mN, pre- and post-ebselen, respectively,

218 n=6, P<0.05) that was not associated with significant hyperpolarization (-44.3±1.0 vs

219 49.0±3.3mV, pre- and post-ebselen, respectively, n=6). A third LOX inhibitor, PD146176 (5 µM),

220 also reduced EDH (27.5±3.1 and 9.8±2.6 mV pre- and post-PD146176 respectively, n=5,

221 P<0.05). PD146176 also reduced spontaneous tone (1.5±0.3 and 0.2±0.1 mN pre- and post-

222 PD146176 respectively, n=5, P<0.05) that was not associated with significant hyperpolarization

223 (-52.1.±3.9 vs -58.7±4.5 mV, pre- and post-PD146176, respectively, n=5). The similar effect of

224 these three structurally different LOX inhibitors to inhibit EDH and abolish the $K_{Ca}2.3$

225 component of this response indicates a role for LOX-derived AA metabolites in EDH.

226

227

228 Previous studies indicate that the LOX-derived HEETA is metabolized by sEH to THETA

229 Inhibition of sEH reduced HEETA metabolism and enhanced EDH to AA suggesting an

230 important role for HEETA in mediating EDH. The inhibitor of sEH, tAUCB (10µM) did not

231 significantly effect EDH to SLIGRL in control vessels (33.5±5.4 vs. 30.8±6.0mV pre- and post-

232 tAUCB, respectively n=5). Following inhibition of $K_{Ca}3.1$ and $K_{Ca}1.1$ with the combination

233 TRAM-34 and iberiotoxin, the residual hyperpolarization to SLIGRL (17.8±2.8mV, n=5) is

234 mediated solely by $K_{Ca}2.3$. Under these conditions, the $K_{Ca}2.3$-mediated hyperpolarization was

235 significantly augmented by tAUCB (29.2±3.6, P<0.05, n=6, Figure 2A, B). This augmented

236 response is specific for $K_{Ca}2.3$ since addition of apamin significantly inhibited EDH (7.0±2.0mV,

237 P<0.05, Figure 2A, B). Therefore, an epoxide-containing LOX metabolite of AA contributes to

238 the $K_{Ca}2.3$ component of EDH and is normally metabolized by sEH.

239

240 *Effect of tAUCB and superoxide scavenging on EDH responses in the presence of L-NAME.*

241 In the presence of the NOS inhibitor L-NAME, inhibition of $K_{Ca}3.1$ alone abolishes EDH . The

242 absence of a $K_{Ca}2.3$ component reflects activation of TP receptors . Similarly, in this study, the

243 isolated EDH-mediated response obtained in the presence of L-NAME (hyperpolarization of

244 23.1±4.6 mV and relaxation of 74.3±5.3%, n=8) was significantly attenuated by TRAM-34 alone

245 (hyperpolarization of 6.5±1.5 mV and relaxation of 25.0±4.2%, n=5; Figure 2C). Application of

246 tAUCB to TRAM-34-treated arteries failed to recover the EDH response (hyperpolarization of

247 4.2±2.4mV and relaxation of 23.7±2.7%, n=5; Figure 2C). In the combined presence of L-

248 NAME and tempol, a cell permeable superoxide scavenger, isolated EDH responses to SLIGRL

249 were significantly inhibited only by the combination of TRAM-34 and apamin. Further inhibition

250 of $K_{Ca}1.1$ with iberiotoxin abolished the residual EDH response (Figure 2D). Thus, tempol

251 restored the $K_{Ca}2.3$ component to the EDH whereas inhibition of sEH failed to restore this

252 component of EDH.

253

254

255 *AA metabolism of the rat middle cerebral artery.*

256 Since LOX metabolites mediate a component of EDH, we investigated the metabolism of ^{14}C-AA

257 by cerebral arteries. In arteries treated with the COX inhibitor, indomethacin, to facilitate the

258 identification of LOX metabolites, the products of AA metabolism were resolved by reverse

259 phase HPLC using solvent system 1. ^{14}C-Labelled metabolites co-migrated with the LOX

260 products THETAs, HEETAs, 15-HETE and 12-HETE (Figure 3A). There were virtually no AA

261 metabolites eluting with the EETs indicating that it is unlikely that the middle cerebral artery

262 produces cytochrome P450 epoxygenase products. In the presence of the NOS inhibitor L-

263 NAME, there was a significant change in the profile of AA metabolism. Metabolites co-migrating

264 with 12-HETE and 15-HETE were markedly increased whereas the metabolites co-migrating

265 with the THETAs and HEETAs increased slightly (Figure 3B).   Arteries were also incubated in

266 the presence and absence of L-NAME and the metabolites resolved by reverse phase HPLC using

267 solvent system 2 that resolves COX metabolites and isoprostanes. Indomethacin was not present

268 in these incubations. Metabolites comigrating with 6-keto $PGF_{1\alpha,}$ 8-iso-PGF_2 and 12-HETE were

269 detected (Figure 4). A ^{14}C-metabolite comigrating with TxB_2 was not observed. Following L-

270 NAME treatment, there was also a marked increase in polar metabolites eluting in fractions 5-10

271 and possibly 8-iso-PGF_2, eluting in fractions 33-34 (Figure 4).   Isoprostane synthesis was

272 confirmed by LC/MS analysis. Extracts were purified by reverse phase HPLC using solvent

273 system 3 and the eluate monitored by selected ion monitoring of m/z 351 for PGE_2-type

274 isoprostanes and m/z 353 for PGF_2-type isoprostanes (Figure 5).   The production of 8-iso-PGE_2

275 was similar in control and L-NAME-treated arteries (380 and 335 ng/ml, respectively; Figure

276 5A), whereas 8-iso-PGF_2 production increased 3-fold with L-NAME treatment (179 and 583

277 ng/ml, respectively; Figure 5B). In addition, several unidentified PGF_2-type isoprostanes were

278 produced by cerebral arteries and were increased by L-NAME (Figure 5B).

279

280   *Effect of 8-isoprostane F_2 on EDH*

281   Since cerebral arteries synthesize isoprostanes, and L-NAME increased their production, we

282   investigated the effects of 8-iso-PGF_2 on vascular tone and EDH.   8-iso-PGF_2 caused

283   concentration-dependent contraction of cerebral arteries that was fully inhibited by the TP

284   receptor antagonist SQ 29,548 (10μM, Figure 6A).

285

286   In control vessels, exogenous application of 8-iso-PGF_2 (1 μM) caused significant depolarization

287   and constriction (Em -49.9±2.5 Vs -40.7±2.5 mV and tension 1.0±0.2 Vs 3.9±0.3 mN pre- and

288   post-8-iso-PGF_2, respectively, n=9, P<0.05) but did not significantly affect SLIGRL-induced

289   EDH (28.0±2.7 vs. 23.7± 2.3mV, pre- and post-8-iso-PGF_2 respectively, n=9; Figure 6B, C).   In

290   the presence of 8-iso-PGF_2, EDH was significantly inhibited by TRAM-34 alone (13.6±2.8 mV,

291   P<0.05, n=5; Figure 6BC) whereas apamin alone had no effect (25.5±5.9mV, n=4; Figure 6C);

292   nor did it have any additional affect in combination with TRAM-34 (9.6±1.1mV, n=9; Figure

293   6C). The residual hyperpolarization was blocked by addition of iberiotoxin (3.2± 0.8mV, n=7;

294   Figure 6C).

295

296   **Discussion**

297   This study is the first to demonstrate that a LOX metabolite(s) of AA, contributes to endothelium-

298   dependent hyperpolarization (EDH) in the rat middle cerebral artery and thus may be the, as yet,

299   unidentified AA metabolite involved in EDH responses in the middle cerebral artery. Following

300   inhibition of NOS with L-NAME, the profile of AA metabolism is altered. There is no change in

301   production of LOX products; however, L-NAME increased 8-iso-PGF_2 production and the

302   production of other iso-PGF_2 isomers. We suggest that the increase in isoprostane formation

303   causes the TP receptor-dependent loss of $K_{Ca}2.3$-mediated component of EDH, a phenomenon we

304   had previously attributed to TxA_2 .

305

306 The findings that 15-LOX metabolites, namely HEETA and THETA, contribute to the EDH

307 response in rabbit arteries lead us to investigate whether these metabolites contributed to EDH in

308 the rat middle cerebral artery. This was of particular interest as EDH responses in this artery have

309 a component dependent upon an unidentified AA metabolite . Analysis of AA metabolism by the

310 middle cerebral artery indicates LOX products are generated whereas epoxygenase metabolites

311 such as EETs are not. In control arteries, EDH induced by SLIGRL-NH$_2$ is significantly

312 attenuated by three structurally distinct inhibitors of LOX enzymes. These findings clearly

313 indicate that EDH in this artery is mediated, at least in part, by LOX metabolites.

314

315 K_{Ca} channels mediate the EDH responses to LOX metabolites; the most likely candidates are

316 either 12-HETE, a 12-LOX metabolite, or HEETA and THETA, 15-LOX metabolites . These

317 metabolites are all produced by the rat middle cerebral artery consistent with the expression of a

318 12/15-LOX. Interestingly, 12-HETE (30 nM) had no effect on membrane potential or tone in

319 cerebral arteries (data not shown). Therefore, the 15-LOX products, HEETA or THETA, are most

320 likely the AA metabolites that contribute to EDH responses in this artery.

321

322 HEETA and THETA stimulate hyperpolarization of rabbit arteries by direct stimulation of

323 $K_{Ca}2.3$. As HEETA is rapidly metabolized by sEH to THETA, it is difficult to distinguish

324 between the activities of these two eicosanoids. In order to ascertain which of these metabolites

325 contributes to EDH, we used a selective sEH inhibitor tAUCB ; tAUCB does not affect total

326 EDH but selectively augments the $K_{Ca}2.3$ component of EDH. As inhibition of sEH prevents the

327 rapid breakdown of HEETA to THETA, it is likely that the HEETA, not THETA, is the 15-LOX

328 product involved in EDH. In rabbit arteries, both these LOX products directly stimulate $K_{Ca}2.3$

329 located on the SMC layer and thus act as diffusible EDHFs. However, such an action is unlikely

330 in the rat middle cerebral artery as $K_{Ca}2.3$ are expressed solely on the endothelium . Thus, it is

331 more likely that HEETA contribute to EDH by acting as an intracellular or autocrine modulators

332 of endothelial $K_{Ca}2.3$, as proposed for other AA metabolites involved in EDH responses such as

333 EETs . More recently, metabolites of AA have also been shown to have effects on transient

334 receptor potential channels evoking subsequent K_{Ca} channel activation and dilation .

335

336 Under the experimental conditions described above, the middle cerebral artery produces NO and

337 the $K_{Ca}2.3$ channel contributes to EDH. However, under conditions where NOS is blocked to

338 observe the EDH response in isolation, the $K_{Ca}2.3$ channel component of hyperpolarization is lost

339 and only $K_{Ca}3.1$ channels contribute to the EDH response . The loss of the $K_{Ca}2.3$ channel

340 component involves stimulation of TP receptors . Since LOX products appear to selectively

341 augment $K_{Ca}2.3$ channels, we postulated that TP-mediated inhibition of this channel may be due

342 to a reduction in LOX metabolites. However, following NOS inhibition, there was actually an

343 increase in the AA metabolites corresponding to HEETA, THETA and HETEs. Inhibition of sEH

344 (a treatment that would be expected to protect HEETA production and thus $K_{Ca}2.3$ channel

345 activation) failed to restore $K_{Ca}2.3$ channel function when NOS was inhibited. Therefore, loss of

346 $K_{Ca}2.3$ function following NOS inhibition does not involve decreased production of LOX

347 metabolites. This finding supports a previous study that demonstrated inhibition of LOX has no

348 effect on EDH responses following inhibition of NOS .

349

350 A potential explanation for the loss of $K_{Ca}2.3$ channel function following NOS inhibition, despite

351 increased production of LOX metabolites, is that TP mediated signaling mechanisms overcome

352 the actions of LOX metabolites. TP receptor stimulation inhibits $K_{Ca}2.3$-mediated responses in

353 control arteries able to synthesize NO by a Rho kinase dependent mechanism . Therefore, we

354 wished to confirm that synthesis of the endogenous activator of TP receptors, TxA_2, is increased

355 following NOS synthase inhibition, as previously suggested . Surprisingly, we did not detect

356 TxA$_2$ production with, or without, NOS inhibition. However, following NOS inhibition there was

357 an increase in production of isoprostanes which also stimulate TP receptors .

358

359 The most likely source of isoprostanes is the non-enzymatic reaction of either free or esterified

360 AA with reactive oxygen species such as superoxide . Due to our sampling technique of

361 measuring isoprostanes in the incubation medium it is likely that we were measuring isoprostanes

362 produced from free AA, (although we cannot eliminate the possibility that esterified isoprostanes

363 were also released into the medium); however the endothelium is the mostly likely source of the

364 isoprostanes as it is the major source of AA metabolites in vascular tissues . Interestingly,

365 superoxide production is high in the cerebral vasculature when compared to systemic vessels due

366 to increased expression of NADPH oxidase . As NO reacts rapidly with superoxide, it is

367 conceivable NOS inhibition in the middle cerebral artery increases free superoxide produced

368 from NADPH oxidase and therefore isoprostane synthesis leading to stimulation of TP receptors

369 and loss of KCa2.3 function. The superoxide scavenger tempol prevented loss of $K_{Ca}2.3$ function

370 following inhibition of NOS supporting this hypothesis. Furthermore, using LC/MS, we detected

371 increased production of 8-iso-PGF$_2$ and several as yet unidentified PGF$_2$-type isoprostanes

372 following NOS inhibition. While the source of superoxide was not investigated in this study a

373 similar increase in isoprostane production following inhibition of NOS has been observed

374 kidneys where it was reversed by tempol and knock out of gp91 phox , we thus speculate that a

375 similar NADPH dependent mechanism is responsible for increased isoprostane production

376 cerebral arteries.   In arteries not treated with a NOS inhibitor, exogenous 8-iso-PGF$_2$ constricted

377 the middle cerebral artery via a TP receptor dependent mechanism and mimicked the ability of

378 other TP receptor agonists   to inhibit the $K_{Ca}2.3$ component of EDH. Thus, increased production

379 of 8-iso-PGF$_2$ following NOS inhibition is likely to cause the loss of the K$_{Ca}$2.3 component of

380 EDH.

381

382 The data are summarized in Figure 7, they indicate the novel finding that LOX metabolites of

383 AA, most likely HEETA, contribute to EDH in the middle cerebral artery and that inhibition of

384 sEH with the selective inhibitor tAUCB increases the K$_{Ca}$2.3 component of hyperpolarization.

385 However, the loss of K$_{Ca}$2.3 function following inhibition of NOS is not restored by tAUCB

386 treatment. Instead, NOS inhibition increases F-type isoprostane production resulting in TP

387 receptor stimulation (Figure 7), the loss of K$_{Ca}$2.3 responses and elimination of the effect of

388 HEETA. These findings may have important implications in diseases associated with loss of NO

389 signaling such as stroke and coronary artery disease where increased isoprostane production has

390 been detected . This pathway may be related to the severity of the disease . Our data indicate

391 that inhibition of sEH may not be an effective treatment for cerebrovascular disease but

392 prevention of isoprostane formation may be beneficial.

393

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535

536   **Figure 1:** Effect of lipoxygenase inhibition on endothelium-dependent hyperpolarization (EDH)

537   evoked by SLIGRL (20µM) in rat middle cerebral arteries that are able to synthesize NO. (A) An

538   original, representative trace showing control EDH (upper panel) and the effect of the non-

539   selective lipoxygenase inhibitor NDGA (1 µM) on EDH (lower panel). Also shown are Bar

540   graphs showing (B) control EDH and the effect of lipoxygenase inhibitors NDGA (C) and

541   ebselen (1µM) (D) as well as the effect subsequent inhibition of $K_{Ca}3.1$ (TRAM-34) $K_{Ca}2.1$

542   (apamin) and $K_{Ca}1.1$ (iberiotoxin) on EDH. Control EDH was only inhibited following inhibition

543   of $K_{Ca}2.3$ and $K_{Ca}3.1$ in the combined presence of apamin and TRAM-34, respectively. NDGA

544   and ebselen significantly inhibited EDH, the residual hyperpolarization was inhibited by TRAM-

545   34 alone, subsequent addition of apamin and IbTx had no significant additional effect. *$P<0.05$

546   indicates a significant difference from control.

547 **Figure 2:** The effect of the sEH inhibitor tAUCB on the $K_{Ca}2.3$ component of endothelium-

548 dependent hyperpolarization (EDH) in rat middle cerebral arteries able to synthesize NO. (A)

549 Original traces of $K_{Ca}2.3$-mediated hyperpolarization obtained in the presence of: the $K_{Ca}3.1$

550 inhibitor TRAM-34 and $K_{Ca}1.1$ inhibitor iberiotoxin (upper trace), the subsequent addition of

551 tAUCB (middle trace) and the combination of tAUCB and the $K_{Ca}2.3$ inhibitor, apamin. (B) Bar

552 graph showing control EDH, KCa2.3 dependent EDH in the presence and absence of tAUCB and

553 further addition of apamin. The $K_{Ca}2.3$-dependent hyperpolarization was significantly smaller

554 than control EDH and was potentiated by tAUCB this hyperpolarization was completely

555 abolished by apamin indicating that it was mediated solely by $K_{Ca}2.3$. (C) **B**ar Graph showing the

556 SLIGRL-induced EDH mediated hyperpolarization (left panel) and relaxation (right panel) in the

557 presence of the NOS inhibitor L-NAME. TRAM-34 significantly attenuated EDH responses and

558 the subsequent addition of t-AUCB had no further effect. Under these conditions, there is no

559 contribution from $K_{Ca}2.3$ channels and tAUCB failed to restore the function of this channel. (D)

560 Bar graphs showing the SLIGRL-induced EDH mediated hyperpolarization (left panel) and

561 relaxation (right panel) in the presence tempol and the NOS inhibitor L-NAME. Tempol restored

562 a $K_{Ca}2.3$ component to the EDH response and also revealed a $K_{Ca}1.1$ component (n=5). *P<0.05

563 indicates a significant difference from control, ΨP<0.05 indicates a significant difference from

564 TRAM-34 + IbTx ($K_{Ca}2.3$ hyperpolarization).

565

566 **Figure 3:** Effect of the NOS inhibitor L-NAME on arachidonic acid metabolism by rat cerebral

567 arteries. Arteries were incubated with ^{14}C-AA and indomethacin in the absence (A) and presence

568 of L-NAME (B). Metabolites were extracted and separated by reverse-phase HPLC, solvent

569 system1. Migration times of known standards are shown on each chromatogram. CPM, counts

570 per minute, THETA, trihydroxyeicosatrienoic acid; HEETA, hydroxyepoxyeicosatrienoic acid;

571 HETE, hydroxyeicosatetraenoic acid; 14,15-EET, 14,15-epoxyeicosatrienoic acid; AA,

572 arachidonic acid.

573

574 **Figure 4:** Effect of L-NAME on arachidonic acid metabolism by rat cerebral arteries. Arteries

575 were incubated with ^{14}C-labeled arachidonic acid in the absence (Control, solid line) and presence

576 of L-NAME (100 mM) (dotted line). Metabolites were extracted and separated by reverse-phase

577 HPLC, solvent system 2. Migration times of known standards are shown on each chromatogram.

578 CPM, counts per minute, PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic

579 acid; AA, arachidonic acid.

580

581 **Figure 5**: Effect of L-NAME on iso PGE$_2$ and iso PGF$_2$ production of rat cerebral arteries.

582 Arteries were incubated with AA and indomethacin in the absence and presence of L-NAME.

583 Metabolites were extracted and analyzed by liquid chromatography-mass spectrometry. Selected

584 ion chromatogram *m/z* 351 (iso PGE$_2$) (C) and *m/z* 353 (iso PGF$_2$) (D). Migration times of known

585 standards are shown on each chromatogram. *indicates a marked change in production following

586 treatment with L-NAME.

587

588   **Figure 6:** Effect of isoprostane $F_{2\alpha}$ (8-iso PGF) on middle cerebral arteries. (A) Concentration-

589   response curve showing constriction produced by 8-iso PGF in rat middle cerebral arteries that

590   were able to synthesize NO. This constriction was completely blocked by the selective TP

591   antagonist SQ 29,543. (B) Original traces showing the effect of pre-incubation of 8-iso PGF2 on

592   EDH evoked by SLIGRL (20μM) in rat middle cerebral arteries that were able to synthesize NO.

593   8-iso PGF_2 did not significantly affect control EDH (upper panel) but subsequent inhibition of

594   KCa2.3 (TRAM-34; 1 μM) virtually abolished EDH (lower panel) the vessel viability was

595   assessed by addition of 15mM K^+ which evokes endothelium-independent hyperpolarization and

596   relaxation (C) Bar graph showing the effect of 8-iso PGF on EDH. Control EDH is unaffected by

597   8-iso PGF, TRAM-34 alone significantly inhibited EDH and apamin is without effect. The

598   combination of apamin and TRAM-34 had no further effect to TRAM-34 alone. The data shows

599   that 8-iso PGF_2 inhibits the $K_{Ca}2.3$ component of EDH normally seen under these conditions.

600   *P<0.05 indicates a significant difference from control. ΨP<0.05 indicates a significant

601   difference from 8-iso-$PGF_{2\alpha}$.

602   **Figure 7:** Schematic diagram showing the role of LOX products on EDH responses in the middle

603   cerebral artery and the proposed effect that inhibition of NOS and the resultant increase in

604   isoprostane production has on the EDH response. **(1)** Under conditions where middle cerebral

605   arteries can synthesize NO, metabolism of AA by LOX produces HEETA which is metabolized

606   by sEH to THETA both can activate $K_{Ca}2.3$ channels; as tAUCB augments the $K_{Ca}2.3$ response

607   HEETA is likely to be the metabolite involved **(2)**. Under these conditions the EDH response also

608   involves activation of $K_{Ca}3.1$ and $K_{Ca}1.1$ channels to produce smooth muscle cell

609   hyperpolarization and relaxation **(3)**. When NO is synthesized **(4)** it rapidly reacts with

610   superoxide production of isoprostanes, when NOS is inhibited **(5)** this inhibitory effect of NO on

611   isoprostane production is reduced (N.B. endothelial cells are the major source of vascular AA

612   metabolites but it is possible NO prevents isoprostane production in SMCs), the subsequent

613   stimulation of the TP receptor **(6)** inhibits the $K_{Ca}2.3$ component of EDH as we have previously

614   reported (McNeish & Garland 2007, McNeish et al. 2012). Sold lines/arrows represent known

615   pathways, dashed lines indicate possible/unconfirmed, pathways green arrows are stimulatory

616   pathways, red lines/text represent inhibitory pathways/blocking agents. EC, endothelial cell;

617   SMC, smooth muscle cell

618

Figure 1

Effect of lipoxygenase inhibition on endothelium-dependent hyperpolarization (EDH) evoked by SLIGRL (20µM) in rat middle cerebral arteries that are able to synthesize NO

A) An original, representative trace showing control EDH (upper panel) and the effect of the non-selective lipoxygenase inhibitor NDGA (1 µM) on EDH (lower panel). Also shown are Bar graphs showing (B) control EDH and the effect of lipoxygenase inhibitors NDGA (C) and ebselen (1µM) (D) as well as the effect subsequent inhibition of KCa3.1 (TRAM-34) KCa2.1 (apamin) and KCa1.1 (iberiotoxin) on EDH. Control EDH was only inhibited following inhibition of KCa2.3 and KCa3.1 in the combined presence of apamin and TRAM-34, respectively. NDGA and ebselen significantly inhibited EDH, the residual hyperpolarization was inhibited by TRAM-34 alone, subsequent addition of apamin and IbTx had no significant additional effect. *P<0.05 indicates a significant difference from control.
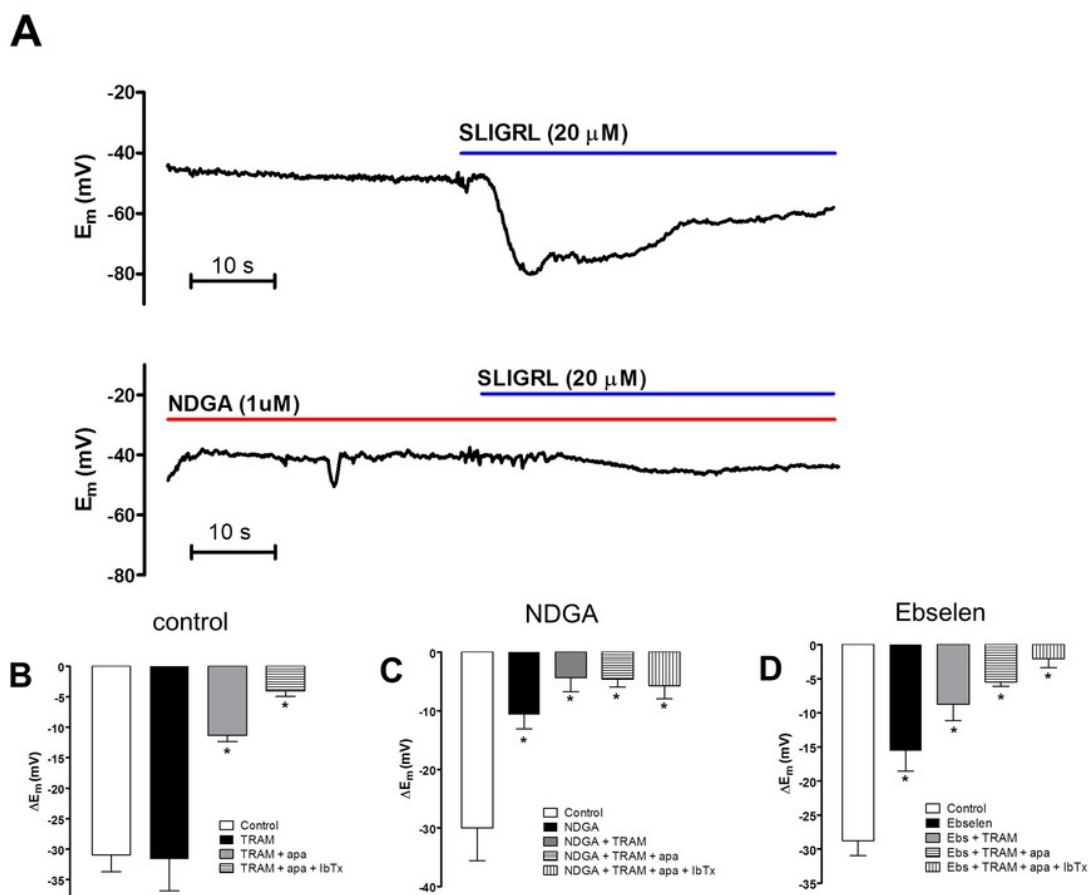
Figure 2

The effect of the sEH inhibitor tAUCB on the $K_{Ca}2.3$ component of endothelium-dependent hyperpolarization (EDH) in rat middle cerebral arteries able to synthesize NO.

(A) Original traces of $K_{Ca}2.3$-mediated hyperpolarization obtained in the presence of: the $K_{Ca}3.1$ inhibitor TRAM-34 and $K_{Ca}1.1$ inhibitor iberiotoxin (upper trace), the subsequent addition of tAUCB (middle trace) and the combination of tAUCB and the $K_{Ca}2.3$ inhibitor, apamin. (B) Bar graph showing control EDH, $K_{Ca}2.3$ dependent EDH in the presence and absence of tAUCB and further addition of apamin. The $K_{Ca}2.3$-dependent hyperpolarization was significantly smaller than control EDH and was potentiated by tAUCB this hyperpolarization was completely abolished by apamin indicating that it was mediated solely by KCa2.3. (C) Bar Graph showing the SLIGRL-induced EDH mediated hyperpolarization (left panel) and relaxation (right panel) in the presence of the NOS inhibitor L-NAME. TRAM-34 significantly attenuated EDH responses and the subsequent addition of t-AUCB had no further effect. Under these conditions, there is no contribution from $K_{Ca}2.3$ channels and tAUCB failed to restore the function of this channel. (D) Bar graphs showing the SLIGRL-induced EDH mediated hyperpolarization (left panel) and relaxation (right panel) in the presence tempol and the NOS inhibitor L-NAME. Tempol restored a KCa2.3 component to the EDH response and also revealed a KCa1.1 component (n=5). *$P<0.05$ indicates a significant difference from control, $P<0.05$ indicates a significant difference from TRAM-34 + IbTx (KCa2.3 hyperpolarization).
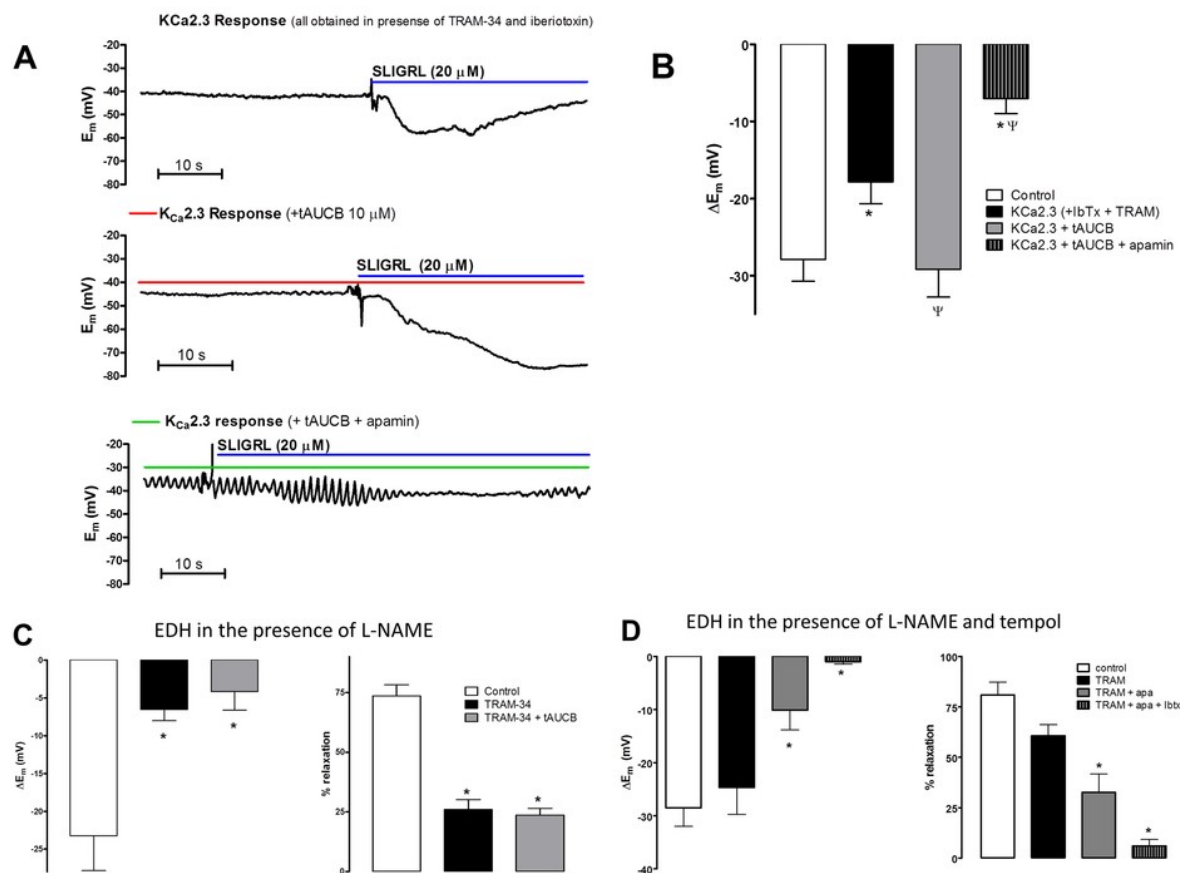
Figure 3

Effect of the NOS inhibitor L-NAME on arachidonic acid metabolism by rat cerebral arteries

Arteries were incubated with ^{14}C-AA and indomethacin in the absence (A) and presence of L-NAME (B). Metabolites were extracted and separated by reverse-phase HPLC, solvent system1. Migration times of known standards are shown on each chromatogram. CPM, counts per minute, THETA, trihydroxyeicosatrienoic acid; HEETA, hydroxyepoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; 14,15-EET, 14,15-epoxyeicosatrienoic acid; AA, arachidonic acid.
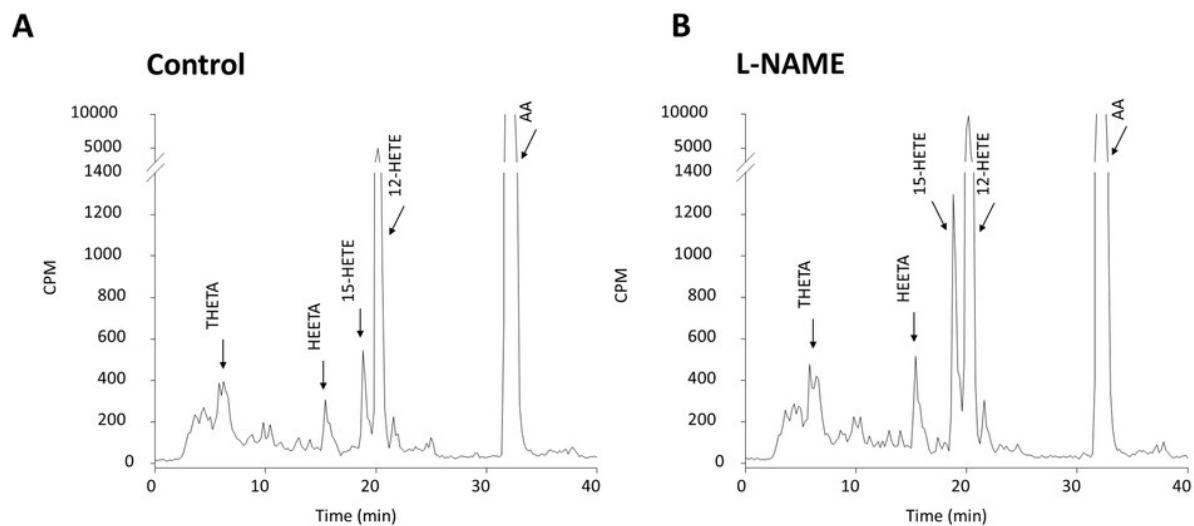
Figure 4

Effect of L-NAME on arachidonic acid metabolism by rat cerebral arteries

Arteries were incubated with ^{14}C-labeled arachidonic acid in the absence (Control, solid line) and presence of L-NAME (100 mM) (dotted line). Metabolites were extracted and separated by reverse-phase HPLC, solvent system 2. Migration times of known standards are shown on each chromatogram. CPM, counts per minute, PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid.
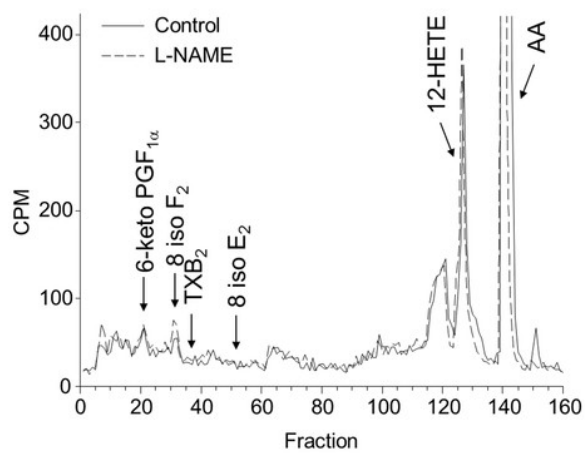
Figure 5

Effect of L-NAME on iso PGE$_2$ and iso PGF$_2$ production of rat cerebral arteries

Arteries were incubated with AA and indomethacin in the absence and presence of L-NAME. Metabolites were extracted and analyzed by liquid chromatography-mass spectrometry. Selected ion chromatogram m/z 351 (iso PGE2) (A) and m/z 353 (iso PGF2) (B). Migration times of known standards are shown on each chromatogram. *indicates a marked change in production following treatment with L-NAME.
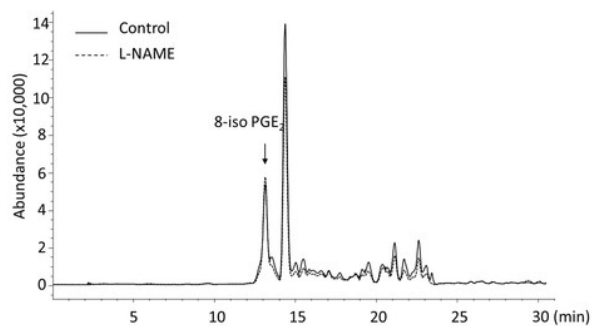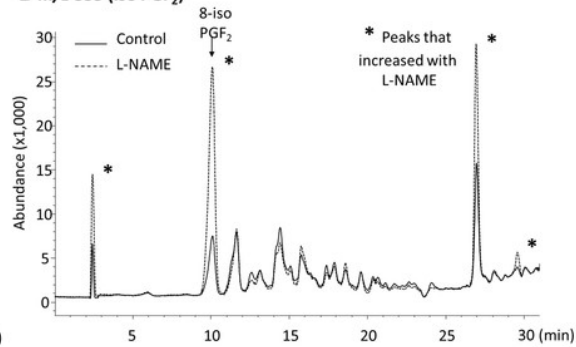
Figure 6

Effect of isoprostane F_{2alpha} (8-iso PGF) on middle cerebral arteries

(A) Concentration-response curve showing constriction produced by 8-iso PGF in rat middle cerebral arteries that were able to synthesize NO. This constriction was completely blocked by the selective TP antagonist SQ 29,543. (B) Original traces showing the effect of pre-incubation of 8-iso PGF2 on EDH evoked by SLIGRL (20µM) in rat middle cerebral arteries that were able to synthesize NO. 8-iso PGF2 did not significantly affect control EDH (upper panel) but subsequent inhibition of KCa2.3 (TRAM-34; 1 µM) virtually abolished EDH (lower panel) the vessel viability was assessed by addition of 15mM K+ which evokes endothelium-independent hyperpolarization and relaxation (C) Bar graph showing the effect of 8-iso PGF on EDH. Control EDH is unaffected by 8-iso PGF, TRAM-34 alone significantly inhibited EDH and apamin is without effect. The combination of apamin and TRAM-34 had no further effect to TRAM-34 alone. The data shows that 8-iso PGF2 inhibits the KCa2.3 component of EDH normally seen under these conditions. *$P < 0.05$ indicates a significant difference from control. $P < 0.05$ indicates a significant difference from 8-iso-PGF2 .
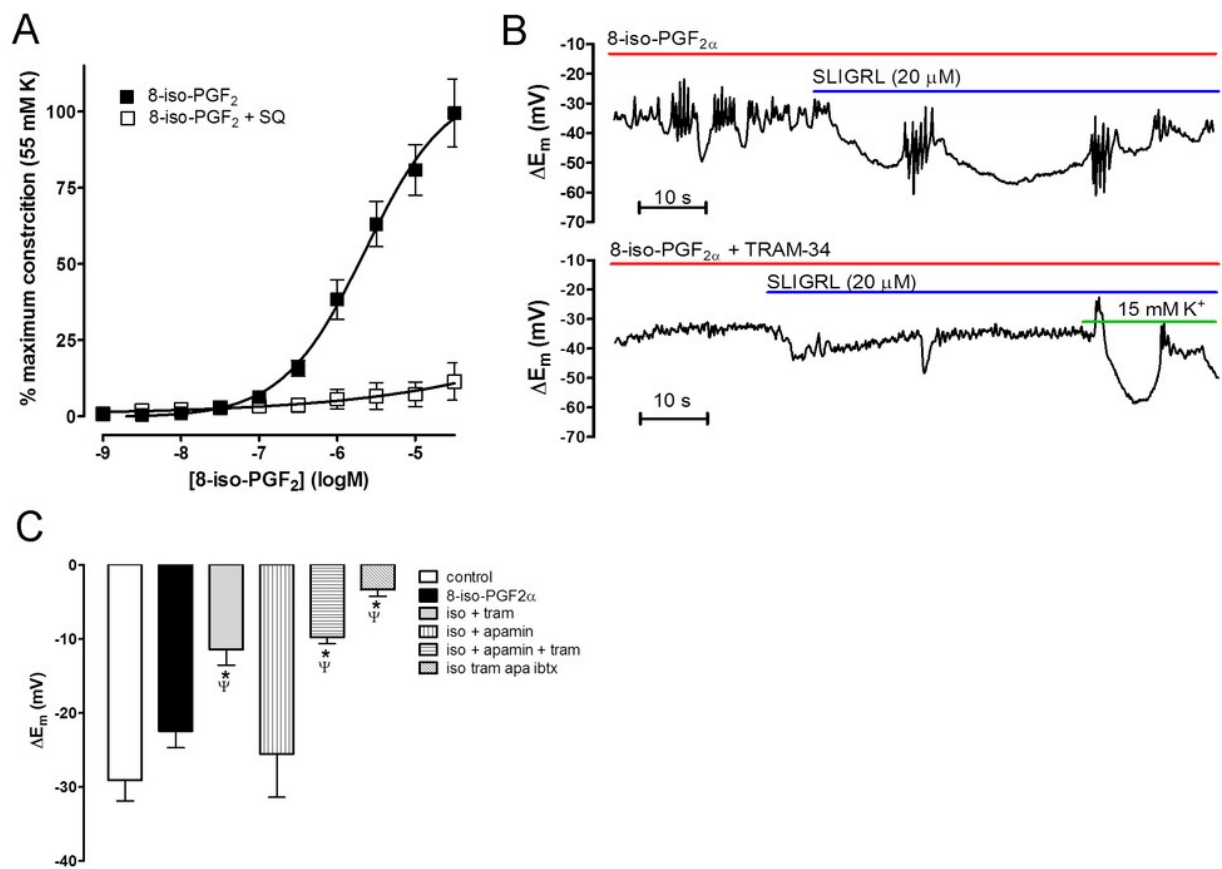
Figure 7

Schematic showing the role of LOX products on EDH responses in the middle cerebral artery, the proposed effect that inhibition of NOS and increase in isoprostane production has on the EDH response

(1) Under conditions where middle cerebral arteries can synthesize NO, metabolism of AA by LOX produces HEETA which is metabolized by sEH to THETA both can activate $K_{Ca}2.3$ channels; as tAUCB augments the $K_{Ca}2.3$ response HEETA is likely to be the metabolite involved **(2)**. Under these conditions the EDH response also involves activation of $K_{Ca}3.1$ and $K_{Ca}1.1$ channels to produce smooth muscle cell hyperpolarization and relaxation **(3)**. When NO is synthesized **(4)** it rapidly reacts with superoxide preventing production of isoprostanes, when NOS is inhibited **(5)** this inhibitory effect of NO on isoprostane production is reduced (N.B. endothelial cells are the major source of vascular AA metabolites but it is possible NO prevents isoprostane production in SMCs), the subsequent stimulation of the TP receptor **(6)** inhibits the $K_{Ca}2.3$ component of EDH as we have previously reported (McNeish & Garland 2007, McNeish et al. 2012). Sold lines/arrows represent known pathways, dashed lines indicate possible/unconfirmed, pathways green arrows are stimulatory pathways, red lines/text represent inhibitory pathways/blocking agents. EC, endothelial cell; SMC, smooth muscle cell