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MEK1/2 inhibition attenuates vascular ET_A and ET_B receptor alterations after cerebral ischaemia

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ABSTRACT

Background and Purpose

Cerebral ischaemia is associated with elevated levels of endothelin B receptors in the ipsilateral middle cerebral artery. This upregulation of endothelin receptors occur via de novo transcription involving mitogen-activated protein kinases (MAPK). The aim of this study was to examine the effect of inhibition of the MAP kinase/ERK kinase (MEK)1/2 on endothelin receptor alteration, brain damage and neurology in experimental cerebral ischaemia.

Methods

Transient middle cerebral artery occlusion was induced in male Wistar rats by the intraluminal filament technique. The animals received 100 mg/kg i.p of the MEK1/2 inhibitor U0126 or vehicle in conjunction with the occlusion. After 24 hours the rats were decapitated and the brains removed. The middle cerebral arteries were dissected out and examined with myographs or immunohistochemistry. The ischaemic areas of the brains were compared.

Results

After the middle cerebral artery occlusion, the contractile responses of the endothelin A and B receptors were augmented in the ipsilateral middle cerebral artery. U0126 decreased this alteration in endothelin receptor response. Furthermore, treatment with U0126 significantly decreased the brain damage and improved neurological scores. Immunohistochemistry showed that there were lower protein levels of phosphorylated extracellular signal-regulated kinases (ERK)1/2 and phosphorylated transcription factor Elk-1 in the U0126-treated rats compared to control.

Conclusions

The results show that treatment with the MEK1/2 inhibitor U0126 in ischaemic stroke decreases brain damage, neurological symptoms and endothelin receptor alteration. The

vascular effects of U0126 provide new perspective on possible mechanisms of actions of MAPK inhibition in cerebral ischaemia.

Keywords: MCAO, ET, MEK1/2, ERK1/2, Elk-1, receptor, ischaemia

INTRODUCTION

Endothelin (ET) is a vasoactive peptide that was first discovered in 1985 by Hickey and colleagues (Hickey et al. 1985). Two endothelin receptors have been identified in mammals, the endothelin type A (ET_A) and type B (ET_B) receptors (Arai et al. 1990; Sakurai et al. 1990). Both are G-protein coupled receptors of which the ET_A receptor is localized on smooth muscle cells in the cerebrovascular wall and stimulates proliferation and vasoconstriction, while the ET_B receptors are mainly situated on endothelial cells, mediating vasodilatation (Nilsson et al. 1997; Szok et al. 2001). The endothelin system has been shown to be involved in several pathophysiological conditions, such as atherosclerosis (Iwasa et al. 1999), heart failure (Miyauchi and Goto 1999) and ischaemic stroke (Ziv et al. 1992; Barone et al. 1994; Lampl et al. 1997). After middle cerebral artery occlusion (MCAO) in cat, there is an enhanced ET mediated constriction in ischaemic vessels (Patel et al. 1996b). Furthermore, an endothelin receptor antagonist increases cerebral blood flow and decreases the ischaemic damage (Patel et al. 1995). This indicates that the endothelin system is involved in limiting the blood flow to the penumbra, an event which might cause a spreading of the ischaemic area. We have previously shown that contractile ET_B receptors are upregulated in the ipsilateral middle cerebral artery (MCA) 48 hours after MCAO (Stenman et al. 2002). To further investigate the mechanisms in this process, we have used organ culture as a model for upregulating ET_B receptors in the rat MCA. This upregulation is time-dependent, reaching a maximum after 24 hours of organ culture (Henriksson et al. 2003), as well as being sensitive to protein kinase C (PKC) inhibitors and inhibitors of the extracellular signal-regulated kinases (ERK) 1/2 pathway (Henriksson et al., unpublished data, 2006; Henriksson et al. 2003; Henriksson et al. 2004).

The ERK1/2 belongs to the family of mitogen-activated protein kinases (MAPK) and are phosphorylated and thereby activated by the MAP kinase/ERK kinase (MEK)1/2. Upon

activation, ERK1/2 induces gene transcription by activating several transcription factors, such as Elk-1, c-fos and c-jun (Seger and Krebs 1995; Whitmarsh et al. 1995; Leppa et al. 1998). Several studies have shown an involvement of the MEK/ERK1/2 signalling pathway in focal cerebral ischaemia, and inhibitors towards this pathway are able to diminish the ischaemic area (Alessandrini et al. 1999; Namura et al. 2001). In addition, Lennmyr and colleagues showed that ERK1/2 is activated in both the ipsilateral and contralateral cerebral blood vessels (Lennmyr et al. 2002).

In the present study we wanted to examine i) if there is an alteration of the vascular ET receptor response 24 hours after transient MCAO, ii) if this alteration is similarly affected by the MEK1/2 inhibitor U0126 as in organ culture, and iii) to examine if MEK1/2 inhibition decreases the ischaemic area and improves neurological status after MCAO. We found that 24 hours after transient MCAO, there is an increased contractile response of both ET_A and ET_B receptors in the ipsilateral MCA. The receptor alterations are attenuated by systemic treatment with the MEK1/2 inhibitor U0126. Immunohistochemistry shows that phosphorylated ERK1/2 and phosphorylated Elk-1 are less abundant in the smooth muscle cells of the ipsilateral MCA in U0126-treated animals compared to control animals. Furthermore, the ischaemic area is diminished and the neurological status of the U0126-treated animals is improved compared to control animals.

MATERIALS AND METHODS

Middle cerebral artery occlusion

Male Wistar Hannover rats (350-400 g) were obtained from Harlan, Horst, Netherlands. The animals were housed under controlled temperature and humidity with free access to water and food. The experimental procedures were approved by the University Animal Ethics Committee (M131-03). MCAO was induced by an intraluminal filament technique,

previously described by Memezawa and colleagues (Memezawa et al. 1992). Briefly, anaesthesia was induced using 4.5% halothane in N_2O:O_2 (70%:30%). The rats were kept anaesthetized by inhalation of 1.5% halothane in N_2O:O_2 (70%:30%) through a mask. A polyethylene catheter was inserted into a tail artery for measurements of mean arterial blood pressure (MAP), pH, pO_2, pCO_2 and plasma glucose. A rectal temperature probe connected to a homeothermal blanket was inserted for maintenance of a body temperature at 37° C during the operation. A laser-Doppler probe (Perimed, Järfälla, Sweden) was fixed on the skull (1 mm posterior to the bregma and 6 mm from the midline on the right side), measuring the blood flow in an area supplied by the right MCA. Thereafter, an incision was made in the midline of the neck and the right common, external and internal carotid arteries were exposed. The common and external carotid arteries were permanently ligated with sutures. A filament was inserted into the internal carotid artery via an incision in the common carotid artery, and further advanced until the rounded tip reached the entrance of the right MCA. The resulting occlusion was made visible by laser-Doppler flowmetry as an abrupt reduction of cerebral blood flow of 75-90%. Immediately after occlusion, the rats were injected intraperitoneally (i.p.) with either 100 mg/kg U0126 (1,4-diamino-2,3-dicyano-1,4 bis[2-aminophenylthio]butadiene; LC Labs, Boston, USA), an inhibitor of both active and inactive MEK1/2,(Favata et al. 1998) dissolved in 0.5 mL dimethyl-sulfoxide (DMSO; Sigma, St Louis, USA) or 0.5 mL DMSO (control). A pilot study was carried out using 10 mg/kg and 50 mg/kg of U0126 (n=3); however, there were no significant changes in infarct volume and these doses were therefore not examined further.

After the injections the rats were allowed to wake up. Two hours after occlusion the rats were reanaesthetized to allow for withdrawal of the filament and thereby achieve reperfusion. Inclusion criteria were a proper occlusion (> 75% reduction of regional blood flow) and

reperfusion as measured by laser-Doppler. Rectal temperature was measured 30 minutes before occlusion and 1 hour after reperfusion.

The rats were examined neurologically before recirculation and immediately before they were sacrificed, 24 hours after MCAO according to an established scoring system (Table 1) (Bederson et al. 1986b; Menzies et al. 1992; Engelhorn et al. 2004).

Infarct volume evaluation

The brains were sliced coronally in six slices of 2 mm which were stained with 1% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, USA) dissolved in physiological saline solution. TTC interacts with the electron transport chain in the inner mitochondrial membrane of viable tissue and is thereby converted to a red dye (Bederson et al. 1986a). The size of the ischaemic infarct volume was calculated using the software program Brain Damage Calculator 1.1 (MB Teknikkonsult, Lund, Sweden). The swelling of the ischaemic hemisphere was approximated by the ratio of the areas of the two hemispheres in the same slice. The ischaemic area values are compensated for this swelling before being used in the volume calculations. The infarct volume is calculated by numerical integration of the ischaemic area of each slice using the trapezium rule and is expressed as percentage of total brain volume in the slices.

Myograph experiments

Mulvany-Halpern myographs (Danish Myo Technology A/S, Aarhus, Denmark) were used for measurements of the contractile properties of the arteries. The arteries were cut into cylindrical segments, approximately 100 µm in diameter, and the endothelium was removed mechanically by rubbing it off with a thread. Proper removal of the endothelium was controlled by monitoring the vascular responses to 10 mmol/L acetylcholine after endothelin-1 (ET-1) precontraction. The segments were mounted on two 40 µm diameter stainless steel

wires and placed in the myographs (Mulvany and Halpern 1977; Högestätt et al. 1983). One of the wires was connected to a force transducer attached to an analogue-digital converter unit (ADInstruments, Hastings, UK). The other wire was attached to a movable displacement device allowing adjustments of vascular tension by varying the distance between the wires. The experiments were recorded on a computer by use of the software program Chart™ (ADInstruments). The segments were immersed in a temperature-controlled (37° C) bicarbonate buffer of the following composition (mmol/L): NaCl 119; $NaHCO_3$ 15; KCl 4.6; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.5 and glucose 5.5. The buffer was continuously gassed with 5% CO_2 in O_2, resulting in a pH of 7.4. The arteries were given an initial tension of 1.2 mN, and were allowed to adjust to this level of tension for 1 hour. The contractile capacity was determined by exposure to a potassium-rich (63.5 mmol/L) buffer with the same composition as the bicarbonate buffer solution except that NaCl was partly exchanged for KCl. Dose-response curves for sarafotoxin 6c (S6c; ET_B receptor agonist) and ET-1 (ET_A/ET_B receptor agonist) were obtained by cumulative application (10^{-12}-$10^{-6.5}$ mol/L). Following S6c administration, the ET_B receptors are desensitized, leaving only ET_A receptors to interact with ET-1 (Lodge et al. 1995). All agonists were purchased from Sigma, St Louis, USA.
The E_{max} values represent the maximum vascular contraction as response to S6c or ET-1 and were calculated as percentage of the contractile response towards 63.5 mM K^+. The pEC_{50} represents the negative logarithm of the concentration which elicits half-maximum response. For each MCA, one to four segments were used, and a mean value was calculated.

Immunohistochemistry

The MCAs were placed onto Tissue TEK (Gibco, Stockholm, Sweden), frozen and subsequently sectioned into 10 μm slices in a cryostat. The primary antibodies used were rabbit anti-phospho-p44/42 MAPK (pERK1/2; Cellsignalling, Santa Cruz, USA) and rabbit

anti-phospho-Elk-1 (Cellsignalling) diluted 1:50. The secondary antibody used was biotin conjugated goat anti-rabbit (JacksonImmunoResearch, Sohan, UK) diluted 1:100. All dilutions were done in PBS with 0.3% Triton-X100 and 5% foetal calf serum. The Vectastain ABC kit (Vector Laboratories, Burlinggame, USA) together with DAB substrate (Rockland, Gilbertsville, USA) were used for detection according to the manufacturers' instructions. Pictures were taken at 40x magnification. As control, only secondary antibodies were used. Four different sections from each MCA were evaluated blindly.

Calculations and statistical analyses

All data are expressed as mean values ± S.E.M. n = number of rats. Statistical analyses between groups were performed with a non-parametric Mann-Whitney test. $P < 0.05$ was considered significant.

RESULTS

Middle cerebral artery occlusion

In all included animals, a proper occlusion and reperfusion was confirmed by laser-Doppler. Immediately before occlusion, temperature, MAP, pH, pO_2, pCO_2 and plasma glucose were measured and all parameters were within normal range. There were no differences between the groups (data not shown). In both groups, the body temperature increased temporarily the first hours after the occlusion, which is a well known phenomenon (Memezawa et al. 1995). The neurological scores differed between the groups; 4.00 in control group compared to 3.00 ± 0.44 in the U0126 treated group ($P < 0.05$, n = 9 in both groups).

Infarct volume evaluation

Analysis of the infarct volume after staining with TTC revealed that treatment with U0126 significantly decreased the size of the ischaemic area as compared to the control group;

control: 25.2 % ± 1.9 % and U0126: 11.0 % ± 2.8 % ($P < 0.01$, n = 6 in each group; Figure 1AB).

Myograph experiments

There were no differences between the K^+-induced contractions in the control group and the U0126-treated group, nor between the right and left MCAs within the groups (data not shown). In the control group, the contractile response towards S6c was significantly increased in the right occluded MCA compared to the left MCA 24 hours after the occlusion. This is in accordance with previous results, which show a similar upregulation in the right MCA compared to the left MCA 48 hours after the occlusion (Stenman et al. 2002). However, in the present study we show that in rats injected i.p. with 100 mg/kg U0126 immediately after the occlusion, the S6c mediated maximum contraction in the right MCA was significantly decreased compared to the right MCA in control rats ($P < 0.05$, Figure 2A, Table 2). Furthermore, the contractile response towards ET-1, mediated by ET_A receptors, was enhanced in the control right MCA, and this increase was attenuated by U0126 treatment ($P < 0.05$, n = 4-6; Figure 2B, Table 2).

Immunohistochemistry

Immunohistochemistry showed a decrease in phosphorylated ERK1/2 protein and phosphorylated Elk-1 protein in the right MCA after treatment with MEK1/2 inhibitor U0126 compared to control animals (n = 3; Figure 3). Figures are representative for the groups. In control MCA, the level of pERK1/2 was low and in contralateral MCA from the MCAO experiments the level of pERK1/2 was not visibly altered (data not shown).

DISCUSSION

The present study demonstrates that in MCAO, the contractile ET_A and ET_B receptors are upregulated in the ipsilateral MCA, a phenomenon associated with enhanced phosphorylation of the smooth muscle cell ERK1/2 and the transcription factor Elk-1. Inhibition of MEK1/2 in conjunction with the occlusion results in smaller infarct volume, improved neurological function. Furthermore, the observed change in endothelin receptors and ERK1/2 and Elk-1 phosphorylation seen in the control group was inhibited with this treatment..

The involvement of the endothelin system in cerebral ischaemia has been widely discussed during the last 15 years. Several studies suggest that increased ET-induced contractility leads to decreased perfusion of the ischaemic area and subsequently an enlargement of the ischaemic core (Asano et al. 1989; Robinson et al. 1990). Selective ET_A receptor antagonists have been proven to increase cerebral perfusion and decrease the ischaemic area after experimental focal ischaemia (Patel et al. 1996a; Dawson et al. 1999). However, in other studies this beneficial effect of treatment with ET_A receptor antagonists could not be verified (Umemura et al. 1995; Bhardwaj et al. 2000).

The ET_A/ET_B receptor antagonist bosentan has been evaluated with various outcomes in ischaemia. Li and colleagues showed that bosentan decreased myocardial and endothelial ischaemic injury in isolated rat heart (Li et al. 1995). Patel and colleagues demonstrated that application of bosentan after MCAO increased arteriolar diameter (Patel et al. 1996b). However, further studies by the same group failed to show any cerebrovascular effects of bosentan in transient global ischaemia (Patel and McCulloch 1996) and permanent MCAO in rat (McAuley et al. 1996). Furthermore, Chuquet and colleagues showed that BQ-788, a selective ET_B antagonist, when administered into the lateral ventricle, exacerbated the ischaemic damage (Chuquet et al. 2002). All in all, the effects of ET receptor antagonism in ischaemia are noticeably diverse.

The present study shows for the first time that there are enhanced contractile responses mediated by ET_A and ET_B receptors in the ipsilateral MCA 24 hours after transient MCAO. In previous studies we have in detail characterized the cerebrovascular endothelin receptors and shown that the upregulation occurs via transcription involving ERK1/2 (Henriksson et al 2004; Stenman et al 2002). Since the MCA receptor upregulation involves not only two different endothelin receptors but could be the effect of intracellular events affecting more than one gene product, a specific receptor antagonist might not be useful in therapy. This theory is strengthened by the fact that the vascular angiotensin (AT) response is enhanced in the ipsilateral MCA after cerebral ischaemia. There is an increase in AT_1 receptor mediated contraction 48 hours after transient MCAO with a concomitant elevation in angiotensin converting enzyme mRNA levels, suggesting an increase in local angiotensin II production (Stenman and Edvinsson 2004).

The basis for our study was to find out if inhibition of the MEK1/2 may alter the outcome of MCAO both in terms of infarct volume and in changes in MCA molecular biology and function. To determine the lowest amount of U0126 necessary for inhibition of MEK1/2 different doses were used in pilot experiments and the infarct volume was measured. 100 mg/kg was concluded to be the lowest dose possible. The effect of MEK1/2 inhibition on infarct volume has been studied by Namura and colleagues. However, they used a different species (mouse), time point (7 days) and did not examine the cerebral vasculature (Namura et al. 2001). In the present study, the question of whether the decrease of the infarct volume is a direct effect of MEK1/2 inhibition in neurons or a more indirect effect due to the normalization of the vascular receptor responses remains to be answered. However, indirectly we observed that the brain tissue surrounding the MCA (Figure 3) did only faintly increase in pERK1/2 activity; a modest increase compared to the MCA pERK1/2 and pElk-1 activation.

However, immunohistochemistry demonstrates a lower expression of both pERK1/2 and pElk-1 protein levels in the ipsilateral MCA (and in surrounding brain tissue) of the treated animals, confirming an inhibition of the MEK/ERK1/2 signalling pathway in the cerebrovascular system. Furthermore, the MCA receptor alterations of the ipsilateral arteries could participate in the development of the infarct and hence their normalization could be part of the mechanisms behind the decrease in ischaemic area following the MEK1/2 inhibition.

In conclusion, this study is the first to show an enhanced endothelin response 24 hours after transient MCAO. Treatment with the MEK1/2 inhibitor U0126 reduces the enhanced contractile response of ET_A and ET_B receptors in the ipsilateral MCA as well as decreases the size of the infarct volume and improves neurological status. Further studies need to prove if the increased cerebrovascular contractility is contributing to an aggravation of the cerebral ischaemia. If so, this provides a plausible explanation for the beneficial effects of treatment with U0126 in cerebral ischaemia.

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TABLES

Table 1. Neurological scores after MCAO. (Bederson et al. 1986b; Menzies et al. 1992)

Score	Interpretation
0	No visible deficits
1	Contralateral forelimb flexion, when hold by tail
2	Decreased grip of contralateral forelimb
3	Spontaneous movement in all directions, but contralateral circling if pulled by tail
4	Spontaneous contralateral circling
5	Death

Table 2. Contractile responses towards S6c and ET-1. E_{max} values are expressed as % of K^+ induced contractions, and pEC_{50} values represent negative logarithm of the concentration that produces half-maximum response. *P < 0.05, **P < 0.01 compared to control RMCA. n = number of rats.

	Control				**U0126**			
	RMCA n= 6		LMCA n= 6		RMCA n= 6		LMCA n= 4	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
S6c	40 ± 11	7.99 ± 0.34	8 ± 5*	7.10 ± 0.16	9 ± 3*	7.12 ± 0.16**	8 ± 5	7.25 ± 0.47
ET-1	320 ± 43	7.87 ± 0.12	221 ± 43*	7.91 ± 0.17	204 ± 14*	8.05 ± 0.09	205 ± 49	7.86 ± 0.12

FIGURE LEGENDS

1A. Infarct volume calculated in TTC stained brain sections at 24 hours after MCAO in control (left bar) and in animals receiving 100 mg/kg of U0126 in conjunction with the operation (% of total volume). Data are given as means + S.E.M. with n = 6. Statistical analysis; **P < 0.01.

1B. Example of TTC staining in corresponding brain sections of a control rat and of a U0126-treated rat. The unaffected brain tissue is coloured red, while the ischemic tissue is white.

2. Contractile responses elicited by adding increasing concentrations of S6c (A) and ET-1 (B) in MCA from control (MCAO) and U0126 treated animals. Both ipsilateral (R=right side MCA) and contralateral (L=left side) MCA were studied. Each point represents mean ± S.E.M. For statistical analysis, see Table 2.

3. Immunohistochemical examination of MCA segments from the ischaemic side 24 hours after MCAO (control) and after MCAO in U0126 treated animals (U0126). The immunostaining for pERK1/2 and pElk-1 protein in the smooth muscle cell layer of the MCA was elevated after MCAO and normalized in animals treated with U0126 (studied in four segments of each MCA and evaluated blindly).

FIGURES

Figure 1A

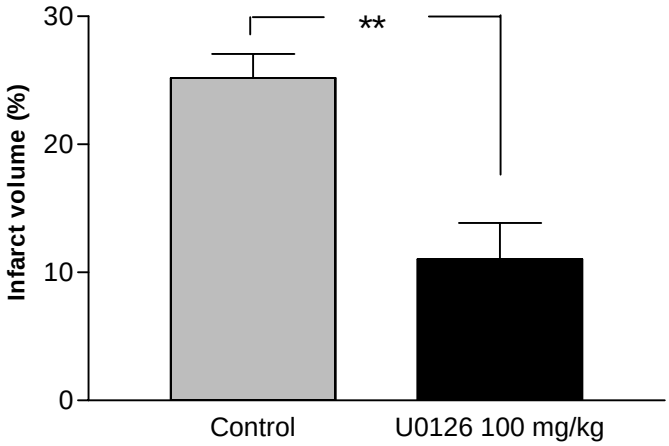
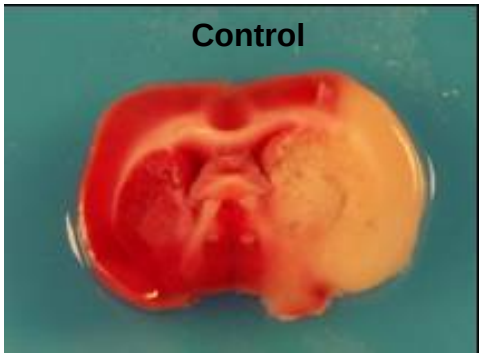


Figure 1B



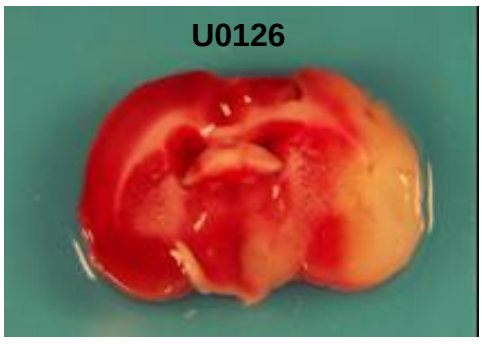


Figure 2A

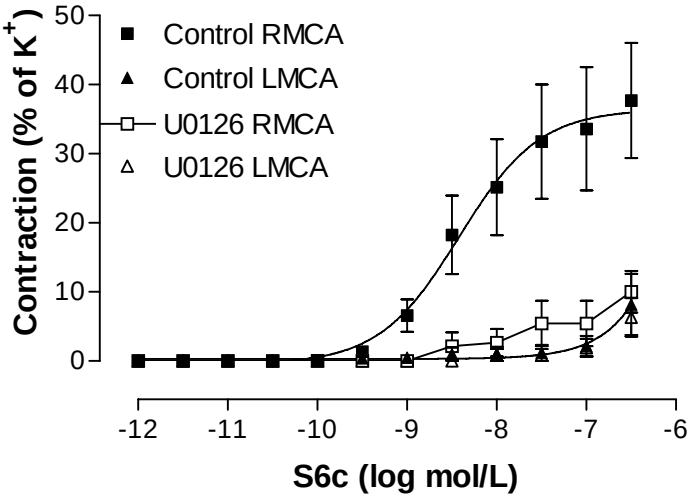


Figure 2B

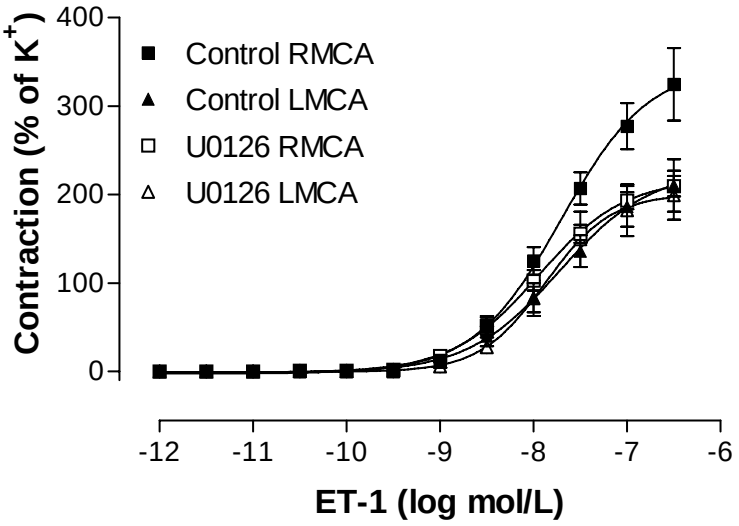


Figure 3