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Up-Regulation of α_{1A}-Adrenoceptors in Rat Mesenteric Artery Involves Intracellular Signal Pathways

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Abstract: The aim of the present study was to investigate if there is an altered expression of α-adrenoceptors during organ culture of rat mesenteric artery segments by using a sensitive pharmacological method and molecular biological techniques. Noradrenalin (NA) induced contraction via α_1-adrenoceptors. The contraction and α_{1A}-adrenoceptor mRNA levels were elevated during organ culture. Transcriptional inhibitor actinomycin D, translational inhibitor cycloheximide, protein kinase C inhibitors (staurosporine and RO31-8220) and mitogen-activated protein kinase (MAPK) pathway inhibitors (SB386023, U0126 and SB239063) prevented the increase in NA-induced contractions. The amount of α_{1A}-adrenoceptor mRNA was significantly lower in the artery segments cultured for 1 day in the presence of specific MAPK extracellular signal-regulated protein kinase1/2 pathway inhibitor SB386023 than that of the cultured controls. SB386023 did not affect α_2-adrenoceptor mRNA level. Our results suggest that the up-regulation of α_{1A}-adrenoceptors involves transcription and intracellular signal transduction via the protein kinase C and the ERK 1/2 pathways.

Introduction

The expression of G-protein coupled receptors (GPCR) is not static but a dynamic process which can alter in pathological states. Increased expression of GPCR may significantly contribute to enhanced vascular tone and development of vascular disease in man. We have recently shown that endothelin A and endothelin B receptor mRNA levels are significantly higher in coronary arteries from patients with ischaemic heart disease than in congestive heart failure or in healthy controls (Wackenfors *et al*. 2004). Furthermore, there is up-regulation of endothelin B receptors in cerebral arteries from patients with cerebrovascular disease (Hansen-Schwartz *et al*. 2002a), in experimental stroke (Stenman *et al*. 2002) and subarachnoid haemorrhage (Hansen-Schwartz *et al*. 2003b). In addition, angiotensin AT_1 receptors

are up-regulated in cerebral ischaemia (Stenman & Edvinsson 2004) and 5-hydroxytryptamine$_{1B}$ (5-HT$_{1B}$) receptors in subarachnoid haemorrhage (Hansen-Schwartz *et al*. 2003a). The up-regulation of several receptor types may explain the limited success in treating cerebrovascular disease using single type of receptor antagonists. So far the endothelin B, AT$_1$ and 5-HT$_{1B}$ receptors have been studied in this regard, but other receptor systems may also undergo changes (Hansen-Schwartz 2004). The α-adrenoceptor plays an important role in cardiovascular disease and is related to vascular contraction in cardiovascular disease. There is increased α-adrenoceptor mediated vasoconstriction in hypertensive patients (Jie *et al*. 1986) and in experimental ischaemia (Sapienza *et al*. 1996) which implies a change in adrenoceptor expression.

Organ culture of vessel segments has been found to be an accessible model to examine phenotypic changes in the smooth muscle cell receptor expression and development of vascular endothelial dysfunction (Adner *et al*. 1995; Alm *et al*. 2002). Rat mesenteric artery has been used as a representative peripheral resistance artery that is able to change the receptor phenotype during organ culture (Adner *et al*. 1996). The present study was designed to examine if there is alteration in the expression of α-adrenoceptors in rat mesenteric artery during organ culture using a sensitive *in vitro* pharmacological method and real-time PCR for receptor mRNA analysis in combination with selective inhibitors of protein kinase C and mitogen-activated protein kinase (MAPK).

Methods

Tissue preparation and organ culture procedure. Sprague-Dawley rats (body weight 250–300 g) were anaesthetized with CO_2 and exsanguinated. The superior mesenteric artery was removed gently, immersed in cold buffer solution and dissected free of adhering tissue under a light microscope. The endothelium was denuded by perfusion of the vessel for 10 sec. with 0.1% Triton X-100 followed by another 10 sec. with buffer solution (see below). The vessels were then cut into 1 mm long cylindrical segments, used directly (fresh group) or incubated for 6 hr to 2 days at 37 ° in humified 5% CO_2 in O_2 (organ culture group). The segments for organ culture were placed in a 96-well plate, one segment in each well, containing 300 µl Dulbecco's modified Eagle's medium containing L-glutamine (584 mg/l) and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). The medium was changed to fresh daily. The protocol was approved by the Ethical Committee of Lund University (M120-01).

In vitro pharmacology. Fresh or cultured segments were immersed in temperature-controlled (37 °) tissue baths containing a bicarbonate buffer solution. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. Each segment was mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer connected to a PowerLab unit (AD instrument, UK) for continuous recording of isometric tension using a PC computer and the software program Chart®. A tension of 2.5 mN was applied to each segment and the segments were allowed to stabilize at this tension for at last one hour before they were exposed to a 60 mM potassium buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the segments were used only if potassium elicited reproducible responses over 0.5 mN. Concentration-response curves were obtained by cumulative administration of noradrenalin (NA).

Buffer solutions and drugs. Standard buffer solution (mM): NaCl 119; $NaHCO_3$ 15; KCl 4.6; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.5; glucose 5.5. Analytical grade chemicals and double distilled water were used for preparing all solutions. Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Gibco BRL, Paisley, Scotland. Noradrenalin, prazosin, rauwolscine, cycloheximide, actinomycin D, staurosporine, RO31-8220, SB386023, SB239063 and U0126 were from Sigma, St. Louis, USA. SB386023, SB239063 and U0126 were dissolved in DMSO.

Real-time reverse transcription (RT)-PCR. Smooth muscle isolated from fresh or cultured segments were homogenized in 1 ml of the RNApro™ solution (Q-BIOgene, CA, USA) by using a FastPrep® instrument (Q-BIOgene, CA, USA). Total RNA was extracted following a protocol from the FastRNA® Pro kit supplier. Reverse transcription of total RNA to cDNA was carried out using the Gene Amp RT kit (PE Applied Biosystems) in a Perkin-Elmer 2400 PCR machine at 42 ° C for 30 min. The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit (PE Applied Biosystems) in a Perkin-Elmer Real-time PCR machine (PE, GeneAmp 5700 sequence detection system). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Specific primers for rat α_{1A} and α_2 receptors were designed based on gene bank data by using primer expression 2 software (PE Applied Biosystems).

α_{1A}-adrenoceptor primers:

Forward: 5'-CTC CAC TGT GCT GCC CTT C-3'

Reverse: 5'-TGC CAA AGG CCC AGT AGC-3'

α_2-adrenoceptors primers:

Forward: 5'-TCA TTG TCA CTG TGT GGG TCA TC-3'

Reverse: 5'-TGA GTG GCG GGA AGG AGA-3'

The house-keeping gene, GAPDH mRNA continuously expressed to a constant amount in the cells, was compared with the house keeping gene elongation factor-1 (EF-1) in a pilot study by real time PCR. GAPDH was used as a reference in this study, but both were equally well constant in the tests.

GAPDH primers:

Forward: 5'-GGC CTT CCG TGT TCC TAC C-3'

Reverse: 5'-CGG CAT GTC AGA TCC ACA AC-3'

The PCR reaction was performed in a 50 µl volume and started at a temperature of 50 ° for 2 min., 95 ° for 10 min. and the following 40 PCR cycles with 95 ° for 15 sec. and 60 ° for 1 min. Dissociation curves were run after the real time PCR to identify the specific PCR products. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems) and synthesized by TAG Copenhagen A/S (Denmark).

Data were analyzed with the comparative cycle threshold (CT) method. To evaluate the amount of adrenoceptor mRNA in a sample, GAPDH mRNA was assessed in the same sample simultaneously. The CT values of GAPDH mRNA were used as a reference to quantity the relative amount of adrenoceptor mRNA. The relative amount of mRNA was calculated with the CT values of alpha receptor mRNA in relation to the CT values of GAPDH mRNA in the sample.

Assay of phosphorylated ERK1/2. Smooth muscle was isolated under cold condition (4 °) from fresh or cultured segments after 0.1% Triton X100 perfusion (Materials and Methods, tissue preparation) and mechanical removal of the adventitial layer, homogenized in 0.5 ml of extraction buffer (BioSource International, Inc. CA, USA) supplied with fresh 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and protease inhibitor cocktail (Sigma) by using the FastPrep® instrument (Q-BIOgene, CA, USA). Measurement of phosphorylated ERK1/2 was performed by using a PhosphoELISAs assay kit (BioSource International, Inc. CA, USA) following the instruction from the supplier. Briefly, the sample was boiled for 5 min. to denature proteins. The sample (100 µl) was added in each pre-coated well and incubated for 2 hr at room temperature. Removal of the sample by aspiration and wash with working wash buffer (supplied in the kit) and then addition of 100 µl of primary antibody for phosphorylated ERK1/2 to each well and incubated at room temperature for 1 hr. Thereafter, the primary antibody was removed by aspiration, washed away and 100 µl of anti-Rabbit IgG-HRP conjugated secondary antibody added to each well and incubated for 30 min. at room temperature. The second antibody was removed and 100 µl of stabilized chromogen was added to each well. The plate was incubated at room temperature for 30 min. in darkness to develop colour. Stop solution 100 µl was added to each well to stop the reaction. The plate was read at an absorbance of 450 nm. The outcome was normalized by the analysis of total protein (Lowry's method).

Calculation and statistics. Calculations and statistics were performed using the GraphPad Prism 3.02 software. Contractile responses in each segment are expressed as a percentage of the potassium-induced contraction. E_{max} refers to maximum contraction calculated as percent of the contractile capacity of 60 mM K^+. pD_2 refers to the negative logarithm of the drug concentration that elicited 50% contraction, which was determined by fitting the data to the Hill equation. Statistical significance was accepted when $P<0.05$, using Student's t-test. All differences referred to in the text have been statistically verified. Values are presented as means± S.E.M.

Results

In vitro pharmacology.

Effect of organ culture on contractile responses. NA induced concentration-dependent contractions of fresh mesenteric artery segments with a maximal contraction (E_{max}) of 169%±8% in relation to 60 mM K^+ and a $pD_2$of 6.66±0.06, respectively. Enhanced contraction was seen after organ culture (fig. 1). The E_{max} values obtained after incubation for 6 hours (n=8), 1 day (n=10) and 2 days (n=10) were 224%±8%, 261%±23% and 225%±10%, respectively, all significantly increased ($P<0.05$) compared with fresh segments (table 1). In vascular segments incubated for 1 day, both the α_1-adrenoreceptor antagonist, prazosin, at low concentrations (10^{-11}M–10^{-8}M) and the α_2-adrenoceptor antagonist, rauwolscine, at

higher concentrations (10^{-7}M~ 10^{-5}M) resulted in a rightward shift of the NA-induced concentration-contraction curves (fig. 2A and B). Schild plot analysis revealed that the pA_2 of prazosin and rauwolscine were 9.65 and 6.42, respectively. A similar result for prazosin and rauwolscine was seen in studies of fresh segments (fig. 2C and D).

Effect of translational and transcriptional inhibitors on NA-induced contractile responses. In artery segments cultured for 1 day with and without the transcriptional inhibitor actinomycin D (5×10^{-5}M) or the translational inhibitor cycloheximide (5×10^{-5}M) showed a depressed NA-induced concentration-contraction response curve (fig. 3). The E_{max} values were reduced from 244±18% to 155±13% (actinomycin D, $P<0.01$) and from 244±18% to 194±10% (cycloheximide, $P<0.05$), respectively (table 2).

The effect of protein kinase C inhibitors on NA-induced contractile responses. Arteries cultured for 1 day with the protein kinase C inhibitor staurosporine or RO31-8220 showed significantly reduced NA-induced E_{max} (fig. 4). The value for E_{max} and pD_2 cultured for 1 day in the presence of staurosporine were (10^{-6}M) 186±19% and 6.26±0.13, respectively, and in the presence of RO31-8220 (10^{-6}M) were 143±26% and 6.15±0.14, respectively (table 2).

Effect of mitogen-activated protein kinase inhibitors on NA-induced contractile responses. NA-induced stronger contraction of mesenteric artery segments cultured for 1 day than in fresh arteries. The concentration-contraction curves in the presence of mitogen-activated protein kinase inhibitors were reduced in the E_{max} as compared to incubated controls (fig. 5). The E_{max} of NA in the presence of SB 386023, U 0126 and SB 239063 were 198±11%, 191±18% and 180±19%, respectively, which all were significantly lower than that of the incubated control E_{max} of 249±20% ($P<0.05$). The pD_2 value for control and either of the different mitogen-activated protein kinase inhibitors did not differ (table 3).

mRNA analysis of α-adrenoceptors.

Real-time RT-PCR analysis was performed on RNA extracted from smooth muscle isolated from the mesenteric arteries that had been treated in the same way as the vessels for *in vitro* pharmacology. The level of α_{1A}-adrenoceptor mRNA was greatly up-regulated after culture for 1 day and this up-regulation was inhibited by SB 386023 (fig. 6). There was no difference in the level of mRNA for α_2-adrenoceptor between fresh and arteries co-cultured with and without SB386023.

Phosphorylation of ERK 1/2.

Phosphorylation of ERK 1/2 was measured with the PhosphoELISA assay kit during organ culture of the mesenteric arteries at 1 and 3 hr after initiation of organ culture. Compared with fresh control, incubation for 1 and 3 hr significantly increased the level of phosphorylated ERK1/2 proteins (fig. 7).

This is the first study to reveal that there is up-regulation of α_{1A}-adrenoreceptors during organ culture of rat mesenteric arteries. The enhanced contraction was confined to an increase in E_{max} with no change in pD_2. Under similar condition, an up-regulation of endothelin B receptors has been shown previously

(Möller *et al*. 2002). The present study showed that in fresh mesenteric arteries the NA-induced contraction was antagonized by prazosin, an α_{1A}-adrenoreceptor antagonist in low concentrations (10^{-11}M~10^{-8}M) and rauwolscine, an α_2-adrenoreceptor antagonist, in high concentrations (10^{-7}M~10^{-5}M). In comparison with results from fresh rat mesenteric arteries, the efficacy of NA on mesenteric arteries after organ culture for one day and 2 days was increased by 52% and 33%, respectively. It has been noted that NA elicited a more efficacy contraction in rat mesenteric arteries cultured for one day than in fresh arteries which agrees well with the results of the present study (Möller *et al*. 1997). The level of α_{1A}-adrenoceptor mRNA was significantly augmented after culture for one day as compared to fresh arteries. The level of α_2-adrenoceptor mRNA did not change. This agrees well with our pharmacological data. The pA_2 value of prazosin 9.65 agrees well with that seen in the literature i.e. pA_2 value 9.35–10.20 for α_1-adrenoceptors (Hussain *et al*. 1997; George *et al*. 2004). The pA_2 of rauwolscine was 6.41 which is lower than that reported before e.g. 8.68 (Paiva *et al*. 1999). This suggests that rauwolscine is not specific for α_2-adrenoceptors but also involved α_1-adrenoceptors in the presently used concentrations (0.1, 1 and 10 μM).

We did not attempt to further delineate the α_1-adrenoceptors functionally into subtypes. From the receptor mRNA study, there was up-regulation of α_{1A}-adrenoceptors, while α_2-adrenoceptors were not significantly altered. This suggests there was an up-regulation of α_{1A}-adrenoceptors after organ culture. Our findings are supported by observations *in vivo* that the contractile responses of skeletal muscle resistance arteries to NA is augmented in patients with critical limb ischemia, which is mediated by α-adrenoceptors and it may aggravate the reduced blood flow to the limbs (Jarajapu *et al*. 2001). The increase in canine femoral artery reactivity, following prolonged ischaemia, seems to be due to an increased density and functional activity of α_{1A}-adrenoceptors expressed by the ischaemic arterial smooth muscle cells (Sapienza *et al*. 1996). There are studies demonstrating increased forearm vasoconstriction to α-adrenoceptor agonists in hypertensives (Jie *et al*. 1986). Therefore we hypothesize that there is α-adrenoceptor up-regulation in some vascular disease as part of the pathophysiology which hence may contribute to the disease progression.

The mechanisms behind the up-regulation of α_{1A}-adrenoceptors was analysed in some details. The enhanced NA-induced contraction during organ culture followed by the elevated levels of α_{1A}-adrenoceptor mRNA suggests a transcriptional mechanism since the enhanced NA-induced contraction was completely abolished if the segments were co-incubated with the transcriptional inhibitor actinomycin D. This supports *de novo* transcription and synthesis of α_{1A}-adrenoceptors during organ culture. A variety of signals (mitogen-activated protein kinase and protein kinase C) facilitate the recruitment of co-activators or of components of the transcription machinery (Rochette-Egly 2003). Organ culture represents a stimulus to the smooth muscle cells, which can activate signal transduction that involves protein kinase C and mitogen-activated protein kinase pathways (Henriksson *et al*. 2003 & 2004). Our study revealed that co-culture for one day with either protein kinase C or mitogen-activated protein kinase inhibitors attenuated the enhanced contraction. The involvement of extracellular signal regulated kinase1/2 (ERK1/2), and p38 protein kinases pathways were demonstrated in experiments using the specific ERK1/2 pathway inhibitor SB386023 and U0126, or the specific p38 protein kinases pathway inhibitor SB239063. All three inhibitor had significant inhibitory effects on the enhanced contractile responses to NA during organ culture. This implies involvement of mitogen-activated protein kinase and protein kinase C pathways in the up-regulation of α_{1A}-adrenoceptors during organ culture. This was further supported by more detailed study since SB2386023 attenuated the up-regulation of

α_{1A}-adrenoceptor mRNA during organ culture. Furthermore, to examine if organ culture possibly involves initiation of ERK1/2 phosphorylation, phosphorylated ERK1/2 protein was studied. We observed that the ERK1/2 protein was phosphorylated at 1 and 3 hr after initiation of organ culture. Since this occurred early it is likely that phosphorylation of ERK1/2 is an early event that leads to the receptor expression. A similar phenomenon in the regulation of endothelin B receptors has been demonstrated in cerebral arteries (Henriksson *et al*. 2004). Understanding the intracellular signal mechanisms for regulation of G-protein coupled receptors might provide a new way for the treatment of vascular disease.

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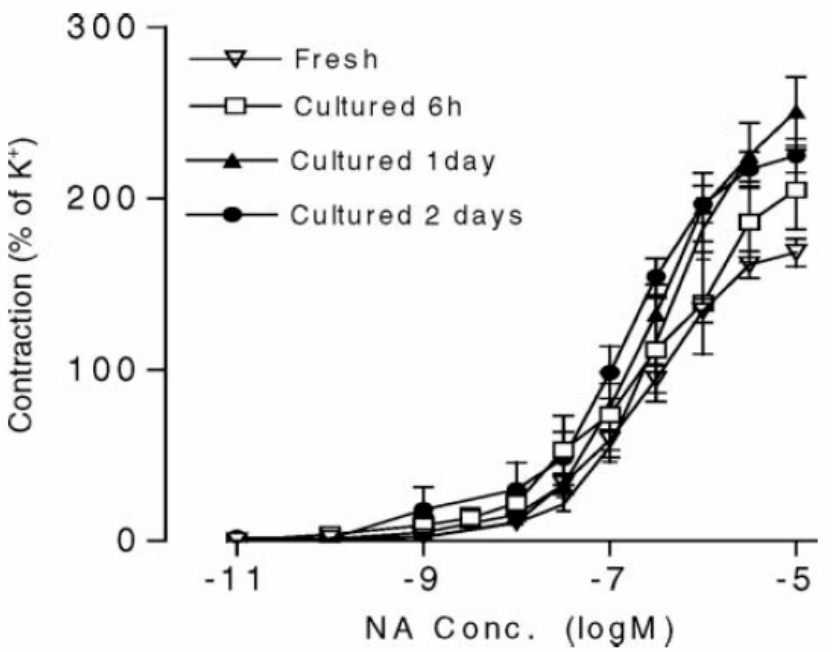

Fig. 1. The contractile effects of NA on fresh and cultured mesenteric arteries. Data are shown as means±S.E.M. n=10.

Table 1.

Effect of NA on fresh and cultured rat mesenteric arterie ring segments without endothelium. Data are the mean±S.E.M. E_{max} are expressed as a percentage of 60 mM K^+-induced contraction. n= number of vessel segments. $^bP<0.01$ and $^aP<0.05$ versus fresh.

	n	K^+ (mN)	E_{max} (%)	pD_2
Fresh	10	2.55±0.44	169±8	6.66±0.06
Cultured 6 hr	8	2.35±0.18	224±22[a]	6.43±0.23
Cultured 1 day	10	2.15±0.18	261±23[b]	6.70±0.10
Cultured 2 days	10	2.12±0.23	225±10[b]	7.09±0.20[a]

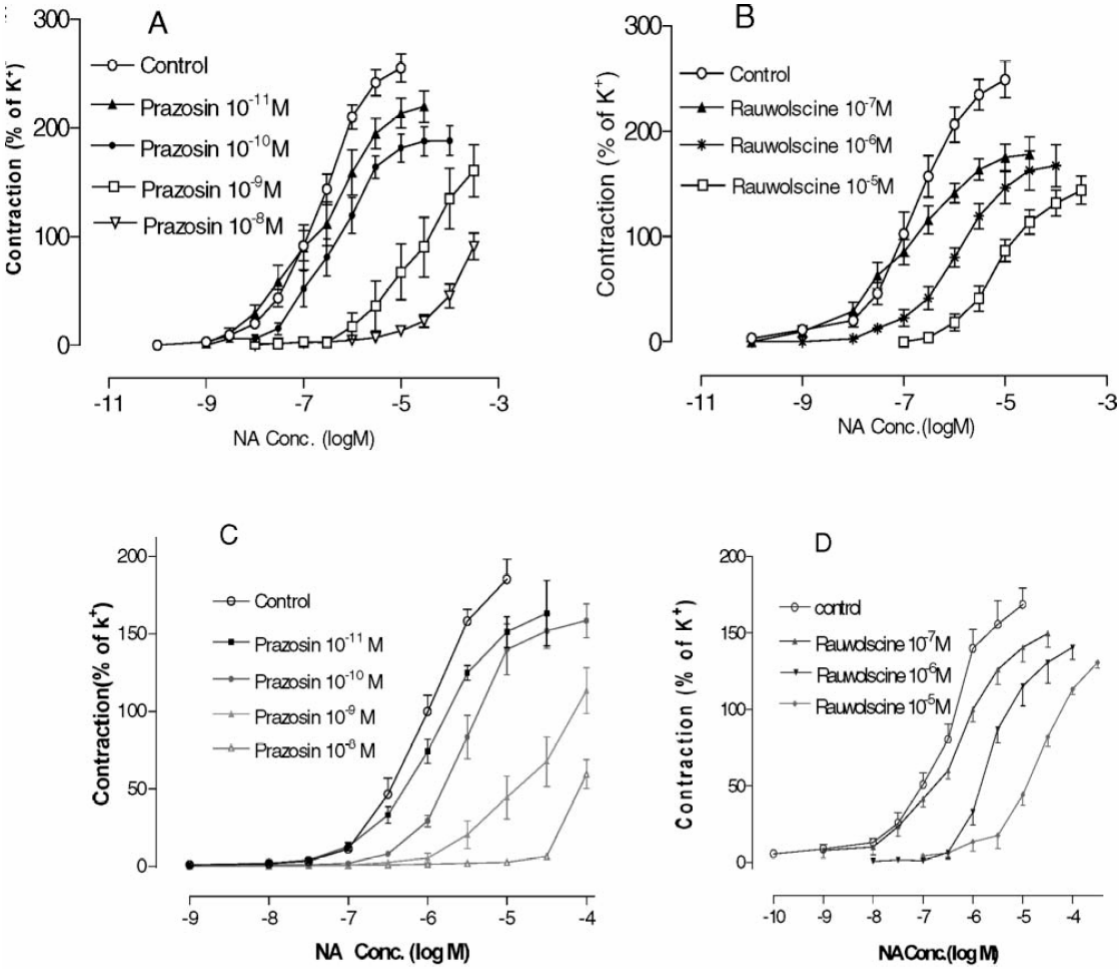

Fig. 2. The contractile effects of NA in the presence and absence of α_1-adrenoceptor antagonist, prazosin 10^{-11}M~10^{-8}M (A and C) or α_2-adrenoceptor antagonist rauwolscine 10^{-7}M~10^{-5}M (B and D) in the fresh mesenteric artery segments (C and D) and the segments after incubation for 24 hr (A and B). Data are shown as means±S.E.M. A: n=9, B: n=10, C and D: n=8.

Table 2.

Effect of translational or transscriptional inhibitor or protein kinase C blocker on up-regulation of NA-induced responses in mesenteric arteries after organ culture for 1 day. Data are the mean±S.E.M. E_{max} are expressed as a percentage of 60 mM K^+-induced contraction. n=number of vessel segments. ^{b}P<0.01 and ^{a}P<0.05 versus incubated 1 day.

	n	K^+ (mN)	E_{max} (%)	pD_2
Fresh	8	2.55±0.51	166±12^b	6.57±0.07
Incubated 1 day	18	2.41±0.16	244±18	6.52±0.11
Incubated with actionmycin D	10	2.32±0.29	155±13^b	6.79±0.09
Incubated with cyclohexmide	10	1.89±0.48	194±10^a	6.34±0.18
Incubated with staurosporine	10	2.47±0.26	186±19^a	6.26±0.13
Incubated with RO 31-8220	10	1.79±0.38	143±26^b	6.15±0.14^a

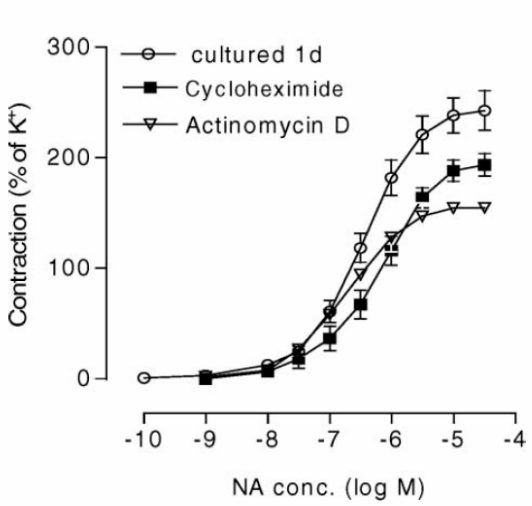

Fig. 3. Effects of NA on the mesenteric arteries cultured for 1 day in the presence and absence of translational inhibitor cycloheximide (5×10^{-5} M) or transcriptional inhibitor actinomycin D (5×10^{-5} M). Data are showed as means±S.E.M. n=10.

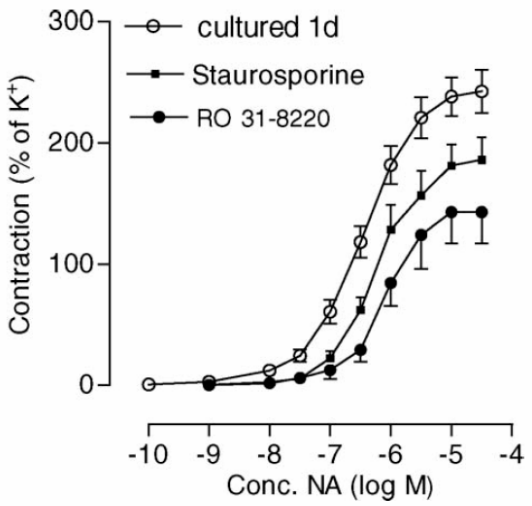

Fig. 4. Effects of NA on the mesenteric arteries cultured for 1 day in the presence of absence or unselective protein kinase C inhibitor staurosporine (10^{-6} M) or specific protein kinase C inhibitor RO 31-8220 (10^{-6} M). Data are showed as means±S.E.M. n=10.

Table 3.

Effect of 3 different MAP kinase inhibitors (each 10 μM) on the enhanced contraction to NA in rat mesenteric arteries cultured for 1 day. Data are the mean±S.E.M. E_{max} are expressed as a percentage of 60 mM K^+-induced contraction. n=number of vessel segments. [a]$P<0.05$ versus incubated 1 day.

	n	K^+ (mN)	E_{max} (%)	pD_2
Incubation 1 day	8	1.24±0.22	249±20	6.77±0.09
SB 386023	8	1.39±0.20	198±11[a]	6.94±0.05
U 0126	8	2.59±0.54	191±18[a]	6.99±0.20
SB 239063	8	1.86±0.46	180±19[a]	6.64±0.07

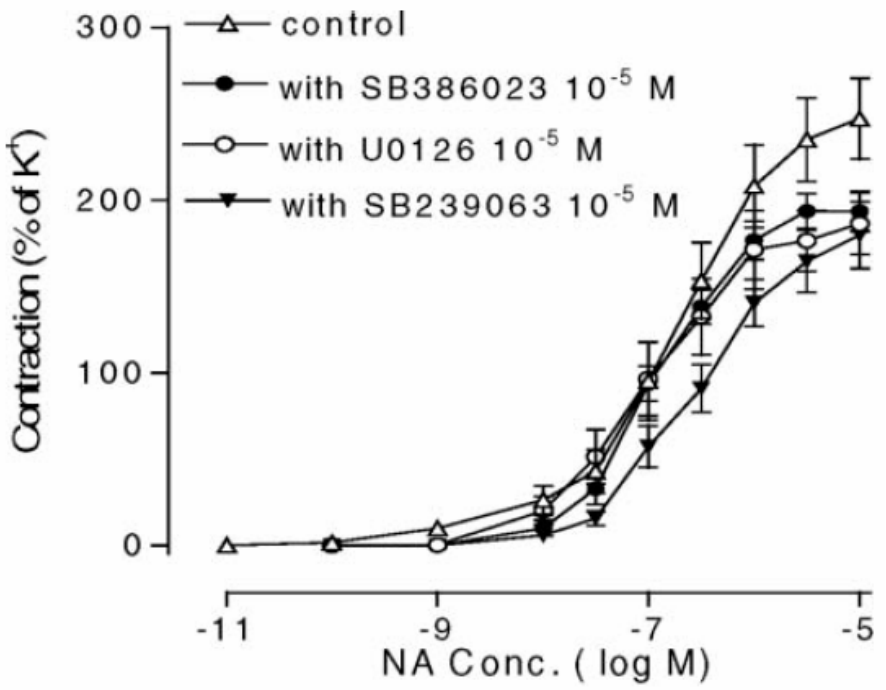

Fig. 5. The effects of NA on the mesenteric arteries cultured for 1 day in the presence or absence of mitogen-activated protein kinase pathway inhibitor SB 386023 (10^{-5} M), U 0126 (10^{-5} M) or SB239063 (10^{-5} M). Data are showed as means±S.E.M. n=10.

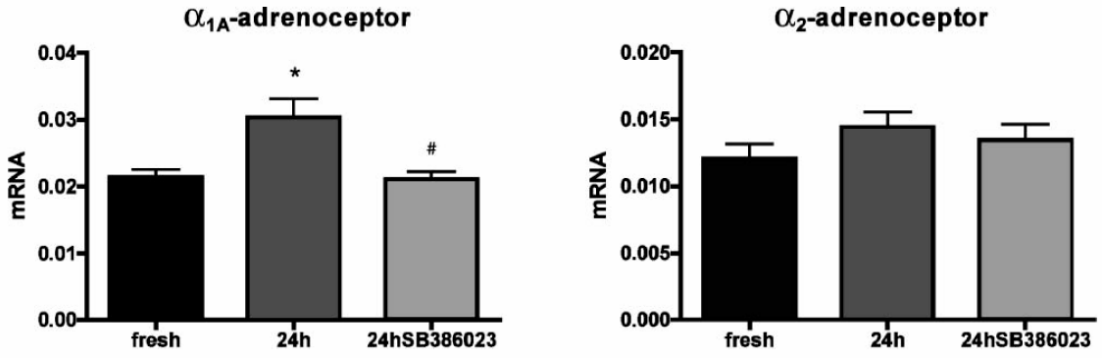

Fig. 6. Quantitative analysis with real-time PCR of mRNA levels for α_{1A}-adrenoreceptors and α_2 adrenoceptors on the mesenteric arteries cultured for 1 day in the presence or absence mitogen-activated protein kinase ERK1/2 pathway inhibitor SB 386023 (10^{-5} M). The bars represent means±S.E.M of three identical experiments. *$P<0.05$ versus fresh, #$P<0.05$ versus cultured for 24 hr.

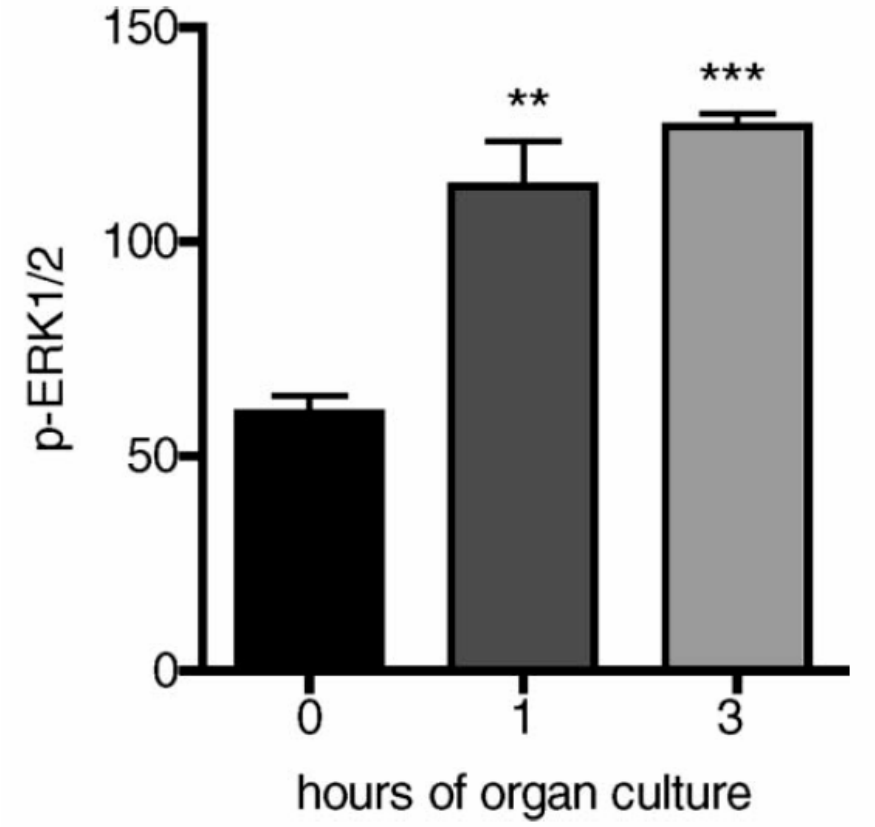

Fig. 7. Phosphorylated ERK 1/2 measured with the PhosphoELISA assay kit during organ culture of the mesenteric arteries at 1 and 3 hr. Three identical experiments were performed and data presented as means with S.E.M. Fresh (organ culture 0 hour) serves as control. **$P<0.01$, ***$P<0.001$ versus control.