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Original article

N-Acetylcysteine-induced vasodilatation is modulated by K_{ATP} channels, Na^+/K^+-ATPase activity and intracellular calcium concentration: An *in vitro* study

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ABSTRACT

Background: In this study, we aimed to investigate the role of ATP-sensitive potassium (K_{ATP}) channel, Na^+/K^+-ATPase activity, and intracellular calcium levels on the vasodilatory effect of *N*-acetylcysteine (NAC) in thoracic aorta by using electrophysiological and molecular techniques.
Methods: Rat thoracic aorta ring preparations and cultured thoracic aorta cells were divided into four groups as control, 2 mM NAC, 5 mM NAC, and 10 mM NAC. Thoracic aorta rings were isolated from rats for measurements of relaxation responses and Na^+/K^+-ATPase activity. In the cultured thoracic aorta cells, we measured the currents of K_{ATP} channel, the concentration of intracellular calcium and mRNA expression level of K_{ATP} channel subunits (KCNJ8, KCNJ11, ABCC8 and ABCC9).
Results: The relaxation rate significantly increased in all NAC groups compared to control. Similarly, Na^+/K^+- ATPase activity also significantly decreased in NAC groups. Outward K_{ATP} channel current significantly increased in all NAC groups compared to the control group. Intracellular calcium concentration decreased significantly in all groups with compared control. mRNA expression level of ABCC8 subunit significantly increased in all NAC groups compared to the control group. Pearson correlation analysis showed that relaxation rate was significantly associated with K_{ATP} current, intracellular calcium concentration, Na^+/K^+-ATPase activity and mRNA expression level of ABCC8 subunit.
Conclusion: Our findings suggest that NAC relaxes vascular smooth muscle cells through a direct effect on K_{ATP} channels, by increasing outward K+ flux, partly by increasing mRNA expression of K_{ATP} subunit ABCC8, by decreasing in intracellular calcium and by decreasing in Na^+/K^+-ATPase activity.

Introduction

Coronary artery disease is the most important cause of morbidity and mortality worldwide and coronary artery bypass surgery is a common option for treating coronary artery disease. Although the surgical procedure is carried out with utmost caution during coronary artery bypass surgery, especially when preparing the arterial grafts, the most important problem is vasospasm. This problem may be relieved by topical or systemic application of relevant pharmacological agents, such as calcium antagonists, nitrovasodilators, papaverine, phosphodiesterase inhibitors, potassium channel openers, and α-adrenoreceptor antagonists.

N-Acetylcysteine (NAC) is a safe and nontoxic a pharmacological agent with generally mild adverse effects. NAC is a precursor in the synthesis of glutathione and acts as an antioxidant, mucolytic,

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and vasodilating substance [1–3]. In particular, NAC acts as a vasodilator in the smooth muscles of the vessels [4,5]. However, the signaling pathway involved in NAC effects is not entirely elucidated. Previous studies have suggested that vasodilatation occurs because NAC elevates NO-mediated cGMP level and decreases the intracellular calcium concentration, involving the endothelium-derived hyperpolarizing factor and prostaglandin I_2 [4–7]. Ceron et al. suggest that the relaxation induced NAC was inhibited by the K^+ channel blockers glibenclamide, selective K_{ATP} channels, and apamin, selective for low-conductance Ca^{2+}-activated K^+ channels in rat aorta, but was not inhibited by charybdotoxin, a potent and selective Ca^{2+}-activated K^+ channel blocker, or by 4-aminopyridine, a voltage-gated K^+ channel blocker [8]. Conversely, Han et al., showed that relaxant effect of NAC was inhibited by 4-aminopyridine, but not by tetraethylammonium and gylbenclamide [9]. K_{ATP} channels are widely expressed in the vascular and visceral smooth muscles, where they play an important role in the regulation of the muscle tone and contraction. These channels contribute to the regulation of the arterial diameter, and are pharmacologically targeted during treatments of various vascular diseases, including hypertension [10]. K_{ATP} channel openers can also activate other K^+ channels. For example, the K_{ATP} channel opener pinacidil can gate calcium-activated K^+ channels [11,12]. Moreover, nicorandil can also induce relaxation of the saphenous vein graft and internal mammary artery *via* K_{ATP} and calcium-activated K^+ channels [13].

The Na^+/K^+-ATPase plays an important role in excitable cells by regulating transmembrane potential, smooth muscle tone, and blood flow [14]. This pump is inhibited by pump inhibitors, such as ouabain, or by the lack of K^+ in the extracellular environment. It has been demonstrated previously that increasing levels of cysteine and glutathione rescued Na^+/K^+-ATPase function and K^+ regulation in patients who took NAC [15].

In the literature, the role of K_{ATP} channels in NAC-induced arterial dilatation is controversial. Additionally, action mechanism of NAC has not been examined on the electrophysiological and molecular levels. In this study, we aimed to establish the mechanism of action of NAC by analyzing of currents of K_{ATP} channels, Na^+/K^+-ATPase activity, intracellular calcium levels, and K_{ATP} channel gene expressions in arterial dilatation.

Material and methods

Experimental animals

Twenty-eight Wistar albino adult male rats ranging between 250 and 300 g in weight were used in this study. The rats were maintained in polycarbonate cages (4 rats per cage) at 25 ± 1.5 °C in the environment with 55% humidity level under the 12:12 h day-night cycle. Standard pellet feed and water were provided *ad libitum*. This study was approved by Mersin University Local Ethics Committee for Animal Experiments. All experiments were carried out in accordance with the National Institutes of Health's (NIH) Guide for the Care and Use of Laboratory Animals.

Chemicals

N-Acetylcysteine was provided as a pure dry powder (Bilim İlaç Sanayi, İstanbul, Turkey).

NAC concentrations

NAC concentrations used were chosen according to previous studies [9,16]. In this study, 2 mM, 5 mM, and 10 mM NAC were used as low, medium, and high concentrations, respectively.

Aortic ring preparations

After rats were anesthetized by an intraperitoneal injection of 75 mg/kg ketamine and 10 mg/kg xylazine, the frontal thorax and abdomen were opened, and the thoracic aorta was isolated. The connective tissue and adipose tissue in the periphery of the isolated tissue were cleaned, and aorta segments were cut into rings of 3–4 mm in length and removed. The two rings that were removed from each animal, one was used for recording relaxation responses, and the other was used to measure Na^+/K^+-ATPase activity.

Tension measurement in rings of thoracic aorta

Rat thoracic aorta ring preparations were divided into four groups as control, 2 mM NAC, 5 mM NAC, and 10 mM NAC. Thoracic aorta rings were transferred to an organ bath that contained 20 mL of the modified Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 0.57 mM $MgSO_4$, 14.2 mM $NaHCO_3$, and 5.5 mM glucose) continuously saturated with a gas mixture of 95% O_2 and 5% CO_2. The organ bath temperature was kept stable at 37 °C by a heating circulator (MAY WBC 3044-PR, Commat Ltd, Ankara, Turkey). One end of the smooth muscle ring was attached to a fixed hook in the organ bath, and the other end was attached to an isometric force transducer (MAY FDT 10-A, Commat Ltd, Ankara, Turkey). After the smooth muscle rings were placed into the organ bath, 2 g of resting muscle tension was applied for 60 min to achieve stability. At the end of the 60 min period, the basal tonus of the muscle was recorded by a Biopac MP100 electrophysiological recording station (Santa Barbara, USA). After basal tonus recording, muscle contraction was achieved by adding 60 mM KCl to the bath solution. This solution was prepared by equimolar substitution of NaCl with KCl in the original Krebs solution. In the experimental groups, when the isometric tension reached the steady level, NAC was added in described concentrations, and the relaxation responses were recorded for 10 min. NAC was not applied to the thoracic aorta rings in the control group. The force signals were amplified and then digitized by a 16 bit A/D converter and stored on a computer for offline analysis. The sampling interval of 50 ms was used in our experiments. The plateau and relaxation values were measured from the records, which were analyzed by AcqKnowledge analysis software (BIOPAC Systems, Inc., Santa Barbara, CA, USA). The percent of relaxation rate (RR) was calculated from the following equation:

Table 1
The thermal cycler program.

	Cycle	Analysis mode	Temperature	Acquisition mode	Time
Initial denaturation	1	None	95 °C	None	00:10:00
Amplification (Quantification)	45	Denaturation	95 °C	None	00:00:10
		Annealing	60 °C	None	00:00:30
		Extension	72 °C	Single	00:00:01
Cooling	1	None	40 °C	None	00:01:00

percent of RR = $[(T_1 - T_2)/T_1]$

Where T_1 is the tension before NAC treatment and T_2 is the tension after NAC treatment.

Na^+/K^+-ATPase activity measurement

Thoracic aorta rings were homogenized on ice with a 50 mM phosphate buffer (pH 7.4) by means of a homogenizer with a Teflon pestle. Then, homogenates were centrifuged at 10,000*g* for 15 min at 4 °C. Supernatants were separated and kept at −20 °C until enzyme activity and protein content measurements were performed.

Na^+/K^+-ATPase enzymatic activity was measured according to the method of Mazzanti et al. [17]. To determine enzymatic activity, solutions with final concentrations of 14 mM KCl, 140 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 40 mM Tris–HCl pH = 7.4, 0.1 mM Na_2EDTA, and 0.2 mM ouabain were added to a sampling tube containing 100 μL of the sample, and then incubated at 37 °C for 10 min. Thereafter, 3 mM ATP was added to each tube to initiate the reaction. After the tubes were incubated at 37 °C for 30 min, the reaction was stopped by the addition of 0.5 mL of 30% TCA to each tube. Inorganic phosphate (Pi) level was measured in the supernatant after centrifugation of the tubes for 10 min. The experimental procedure was repeated without ouabain in the medium. Activity was calculated by subtracting the value of Pi level measured in the presence of ouabain from the value of Pi level measured in the absence of ouabain. The results were expressed as μmol of Pi released per h per mg of protein.

Tissue protein content was measured by a procedure similar to that documented by Lowry et al. using bovine serum albumin as standard [18].

Cultured rat thoracic aorta smooth muscle cell

Rat thoracic aorta smooth muscle cells were obtained from Lonza (R-ASM-580). They were grown at 37 °C in a humidified 5% CO_2 atmosphere in DMEM F12 medium supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and 1% amphotericin (pH range: 7.0–7.4). Cultured cells were used to record K_{ATP} channel currents, to measure intracellular calcium concentration, and to determine mRNA expression levels.

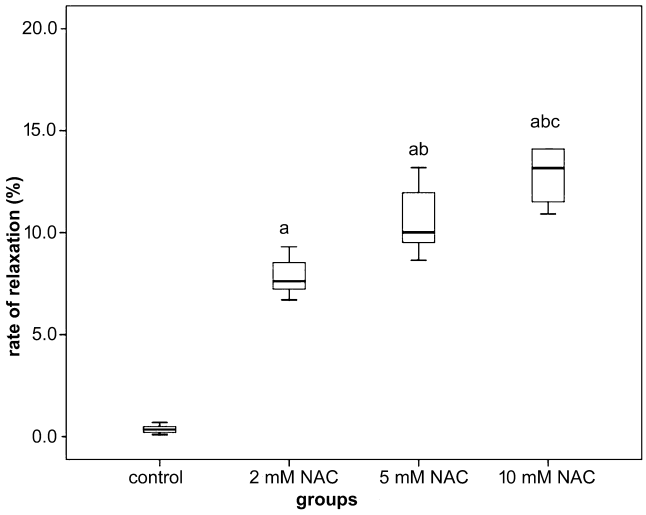

Fig. 1. Relaxation rates (%) in the control and experimental groups. The relaxation rate was concentration dependently increased by NAC in the experimental groups relative to its value in the control group. [a]When compared to the value in the control group, $p < 0.05$. [b]When compared to the value in the 2 mM NAC group, $p < 0.05$. [c]When compared to the value in the 5 mM NAC group, $p < 0.05$.

Recordings of K_{ATP} channel currents

In this study, the patch-clamp method was used to record single-channel currents from the cell membrane in the cell-attached mode [19]. For this purpose, filamented borosilicate glass pipettes were used (World Precision Instruments, USA). The patch pipettes were pulled with a programmable automatic pipette puller (P97, Sutter Instruments, Novato, CA, USA). The resistance of pipettes ranged from 2 to 6 MΩ. The bath solution contained 140 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5.5 mM D-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), whereas the pipette solution contained 140 mM KCl, 5.5 mM D-glucose, and 10 mM HEPES (pH 7.2). Pipette voltage was set to +50 mV, and the experiments were carried out at 37 °C.

Single-channel currents were recorded with a Multiclamp 700B amplifier (Axon Instruments, USA), low-pass filtered at 1 kHz, and digitized at 10 kHz by a Digidata 1322 board (Axon Instruments, USA). Cells and pipettes were visualized by an inverted microscope (BM-37XB, U-Therm International (H.K.) Limited). Current recordings of 1-min duration were analyzed with Clampfit 10 software (Axon Instruments, USA). Current amplitudes were determined by fitting the single-channel current distribution histograms to a Gaussian distribution as below:

$$f(x) = \sum_{i=1}^{n} A_i \frac{e^{-(x-\mu_i)2}/2\sigma_i^2}{\sigma_i^2\sqrt{2\pi}} + C$$

When this function is solved for the amplitude A, the median current value corresponds to the standard deviation and the constant C. This function is commonly used to identify amplitude distributions in single-channel records [20].

Measuring the intracellular calcium concentration

A confocal laser-scanning microscope was used to measure changes in the intracellular calcium concentrations by using Fluo-4AM (Thermo Fisher Scientific Cat #F14201). In brief, cultured vascular smooth muscle cells were incubated with 200 μM Fluo-4 AM at 37 °C for 50 min. Next, Fluo-4 AM loaded cells were allowed to settle on a confocal laser scanning apparatus. Fluo-4 fluorescence was excited with the 488 nm line of the argon laser and emitted fluorescence at 510 nm was detected. The mean fluorescence intensity value for each study group was calculated and converted into a time-course chart.

Quantitative real-time polymerase chain reaction

RT-qPCR was used to determine mRNA levels of KCNJ8, KCNJ11, ABCC8, and ABCC9 genes in the thoracic aorta culture cells. In total, 12 samples of cultured vascular smooth muscle cells were used (three samples per each of the four groups). The minimal number of samples was determined by using statistical power analysis.

Isolation of mRNA

Isolation of mRNA was performed from approximately one million thoracic aorta culture cells from each sample by using a High Pure RNA Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA) and a MagNA Pure Compact instrument (Roche Diagnostics, Indianapolis, IN, USA). Before the samples were loaded into the instrument, 10^6 cells were suspended in a mixture of 200 μL PBS

Table 2
Na^+/K^+-ATPase activity in control and NAC groups.

Variable	Control	2 mM	5 mM	10 mM
Na^+/K^+-ATPase activity (μmol Pi released h^{-1} mg $protein^{-1}$)	0.32 ± 0.024	0.26 ± 0.028[a,b]	0.26 ± 0.027[a,b]	0.20 ± 0.021[a]

[a]When compared to the value in the control group, $p < 0.05$, [b]when compared to the value in the 10 mM group, $p < 0.05$.

and 400 μL of the lysis binding buffer. The subsequent steps were carried out according to the manufacturer's instructions.

Synthesis of cDNA

Total mRNA isolated from the thoracic aorta culture cells was immediately turned into cDNA by using reverse transcription with random hexamer primers. The synthesis of cDNA was performed in two stages using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). During the first stage, for each sample, 9 μL of pure total RNA, 2 μL of a random hexamer primer, and 2 μL of PCR-grade water were mixed and incubated in a thermal cycler at 65 °C for 10 min. During the second stage, 13 μL of the product from the first stage was mixed with 4 μL of the transcriptor reverse transcriptase reaction buffer, 0.5 μL of protector RNase inhibitor, 2 μL of the deoxynucleotide mix, and 0.5 μL of transcriptor reverse transcriptase solution. To obtain cDNA, this mixture was incubated at 25 °C for 10 min, then at 50 °C for 60 min, and at 85 °C for 5 min in the thermal cycler, whereupon it was stored at −20 °C until use.

Real-time PCR

Expression of the *Actb* (beta actin) gene was used as a housekeeping gene benchmark for relative quantifications of the ABCC8, ABCC9, KCNJ8, and KCNJ11 target genes. A Light Cycler 480 Real-Time PCR system (Roche Diagnostics, Indianapolis, IN, USA) was used. Each reaction consisted of 10 μL of the Light Cycler 480 Probe Master mix, 1 μL of Primer Probe, 4 μL of water, and 5 μL of cDNA solutions. The thermal cycler program is shown in Table 1.

The measurements were made at the FAM wavelength of 465–510 nm. The results were calculated by using the "Advanced Relative Quantification" calculation settings and $\Delta\Delta C_T$ method.

Statistical analysis

Statistical analysis was performed with SPSS software version 17.0. After testing for the normality of distribution of experimental values by using the Kolmogorov–Smirnov test, the data were analyzed with the one-way analysis of variance (ANOVA) test. Multiple comparisons were performed with the LSD *post-hoc* test. Pearson correlation method was used for the quantify association between the relaxation rate and K_{ATP} current, decrease of intracellular calcium, Na/K ATPase activity and mRNA expression level of K_{ATP} subunits. Scatter plots were created as part of linear regression analysis. Results were considered significant at $p < 0.05$. All results are presented as mean ± standard deviation.

Results

Thoracic aorta relaxation responses

The thoracic aorta contraction (induced by 60 mM KCl) and relaxation responses were recorded at each NAC concentration. The relaxation rate calculated from the recordings comprised 7.36 ± 1.83% at 2 mM NAC, 10.90 ± 2.39% at 5 mM NAC, and 13.84 ± 2.83% at 10 mM NAC, respectively. Thus, the relaxation rate was significantly and concentration-dependently increased by NAC in the experimental groups relative to its value in the control group ($p < 0.05$; Fig. 1).

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was significantly decreased by NAC in all experimental groups compared to its level in the control group (Table 2). However, although there was no statistical significant difference in Na^+/K^+-ATPase activity levels between the 2 mM and 5 mM groups, level of Na^+/K^+-ATPase activity was significantly

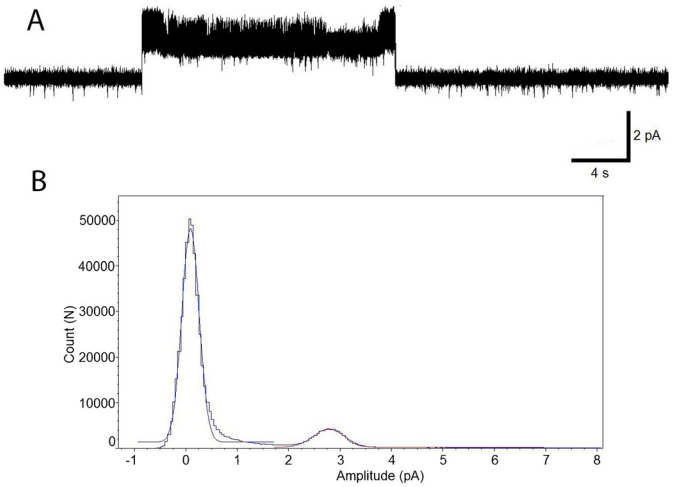

Fig. 2. An example of single channel current records of K_{ATP} (A) and distribution of all points fitted amplitude histogram (B) from the control group. The current level jumps between two average values as the channel opens (upper level) and closes (lower level).

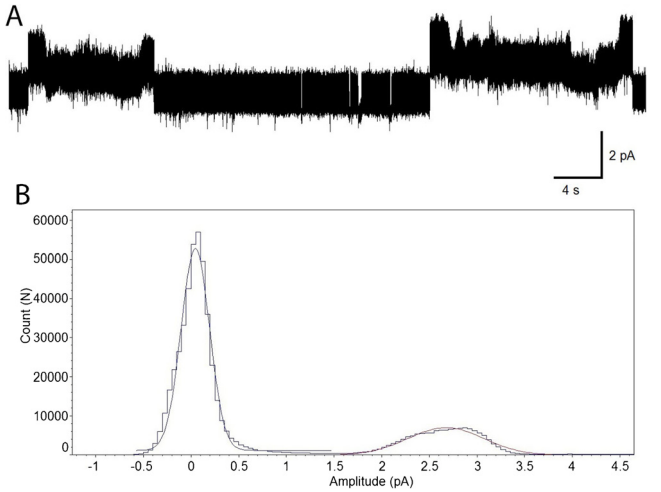

Fig. 3. An example of single channel current records of K_{ATP} (A) and distribution of all points fitted amplitude histogram (B) from the 2 mM NAC group. The current level jumps between two average values as the channel opens (upper level) and closes (lower level).

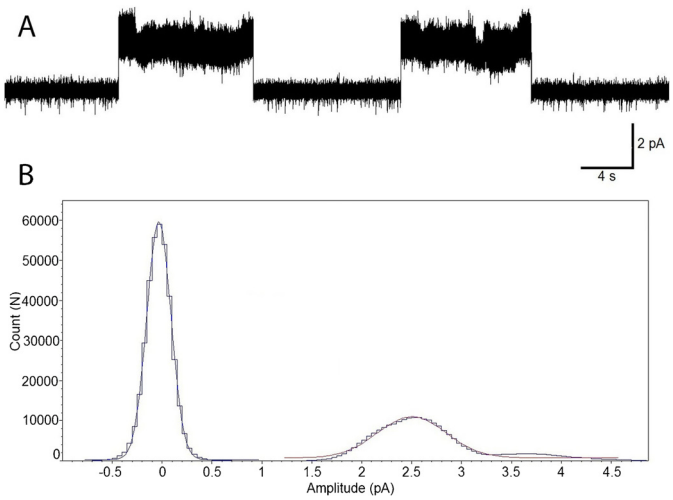

Fig. 4. An example of single channel current records of K_{ATP} (A) and distribution of all points fitted amplitude histogram (B) from the 5 mM NAC group. The current level jumps between two average values as the channel opens (upper level) and closes (lower level).

lower in the 10 mM NAC group than in the 2 mM and 5 mM NAC groups ($p < 0.05$).

Single channels recordings, K_{ATP} currents

Typical recordings of single K_{ATP} channel currents and current histograms for smooth muscle cells from control and each experimental group are illustrated in Figs. 2–5 . The mean current amplitudes were 2.47 ± 0.017, 2.98 ± 0.522, 3.09 ± 0.41, and 3.06 ± 0.31 pA in the control, 2 mM NAC, 5 mM NAC, and 10 mM NAC groups, respectively. In all treatment groups, single channel current amplitudes were significantly larger than that in the control group ($p < 0.05$). There were no statistically significant differences between mean current amplitudes in different NAC groups. The mean current distribution curve is presented in Fig. 6.

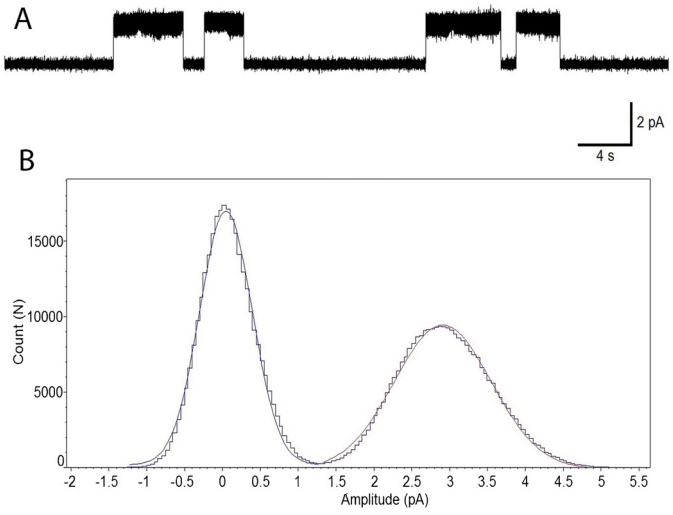

Fig. 5. An example of single channel current records of K_{ATP} (A) and distribution of all points fitted amplitude histogram (B) from the 10 mM NAC group. The current level jumps between two average values as the channel opens (upper level) and closes (lower level).

Intracellular calcium concentration

In the end of a 10-min observation period, the decrease in the intracellular calcium concentration comprised 1.73% in the control group, whereas larger decreases by 22.15%, 49.99%, and 69.53% were detected in 2 mM NAC, 5 mM NAC, and 10 mM NAC groups, respectively. Compared to the amplitude of change in the control group, decreases in the 5 mM and 10 mM NAC treatment groups were significantly higher ($p < 0.05$). Within the treatment groups, decreases in the intracellular calcium concentration elicited by 5 mM and 10 mM NAC were statistically higher than that caused by 2 mM NAC. However, there was no statistically significant difference between changes in the intracellular calcium concentration caused by 5 mM and 10 mM NAC. The relationship between the concentration of NAC and decrease of the intracellular Ca^{2+} concentration is shown in Fig. 7.

mRNA expression of K_{ATP} subunits in rat thoracic aorta

mRNA expression data are shown in Table 3. mRNA expression level of ABCC8 was significantly increased by 2 mM, 5 mM and 10 mM NAC compared to that in the control group ($p < 0.05$). In contrast, the mRNA expression level of ABCC9 was significantly decreased by 2 mM NAC compared to the corresponding values in all other groups ($p < 0.05$). The mRNA expression level of KCNJ8 was significantly decreased by 2 mM NAC ($p < 0.05$), whereas expression of KCNJ11 mRNA was not affected by the NAC treatment ($p > 0.05$).

Association with relaxation rate and K_{ATP} current, intracellular calcium level, Na^+/K^+-ATPase activity and mRNA expression level of K_{ATP} subunits

To measure whether the relaxation rate was correlated with the K_{ATP} current, intracellular calcium concentration, Na^+/K^+-ATPase activity and mRNA expression level of K_{ATP} subunits, a correlation analysis was performed. The results of Pearson correlation analysis showed that there was a significant correlation of relaxation rate versus K_{ATP} channel current ($r = 0.477$, $p < 0.05$), % decrease of intracellular calcium concentration ($r = 0.921$, $p < 0.01$) and Na^+/K^+-ATPase activity ($r = -0.624$, $p < 0.01$). The scatter plots for these significant correlations were shown in Figs. 8–10 , respectively.

The correlation between relaxation rate and mRNA expression level of K_{ATP} subunits is also calculated. While there was a significant correlation between relaxation rate and mRNA expression level of ABCC8 subunit ($r = 0.808$, $p < 0.05$), the correlation between relaxation rate and mRNA expression levels of KCNJ8, KCNJ11 and ABCC9 subunits was not significant ($r = -0.429$, $p > 0.05$; $r = -0.332$, $p > 0.05$; $r = -0.255$, $p > 0.05$, respectively).

Discussion

In this study, we studied the role of K_{ATP} channels, Na^+-K^+ ATPase and intracellular calcium in NAC-induced vasodilatation of thoracic aorta smooth muscle cells using electrophysiological and molecular techniques. Our findings suggest that NAC relaxes vascular smooth muscle cells through a direct effect on K_{ATP} channels, by increasing outward K^+ flux and partly by increasing mRNA expression of K_{ATP} subunit ABCC8 by decreasing in intracellular calcium and by decreasing Na^+/K^+-ATPase activity.

In the present study, activity of the K_{ATP} channels is determined by both single channel amplitudes and channel density of thoracic aorta cells. Amplitudes of single channel current are important for two main reasons. First, the way the amplitude varies with ionic composition of the bathing medium and with membrane potential are important for the study of ion permeation mechanisms.

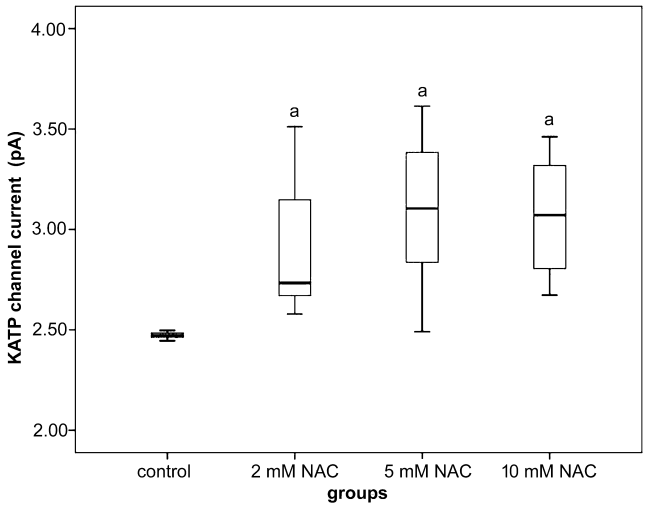

Fig. 6. Values of the mean single channel current amplitude in control and experimental groups. [a]When compared to the value in the control group, $p < 0.05$.

Second, amplitude measurements a useful way to characterize channel types [21]. In our study, we demonstrated that NAC concentration-dependently increased amplitude of K_{ATP} channel current by analysis of single channel records and K_{ATP} channel current was significantly correlated to the relaxation rate. Increased channel activity leading to hyperpolarization due to outward K^+ flow from the cell, which, in turn, resulted in the relaxation of the thoracic aorta smooth muscles. According to our knowledges, there is no study to determine the effect of NAC on the activity of K_{ATP} channel by using single channel recording. Ceron et al. incubated aorta rings with K^+ channel blockers to determine their inhibitory effect on K^+ channels and measured relaxation [8]. They found that incubation with the Ca^{+2} activated K channel blocker, charybdotoxin, did not alter the relaxation induced by NAC. Similarly, the voltage gated K channel blocker 4-amino-pyridine had no inhibitory effect on the relaxation induced by NAC. However, K_{ATP} channel blocker glibenclamide reduced the sensitivity to NAC [8]. Our results are consistent with the results reported by Ceron et al. but differs from that of Han et al., who showed that the ATP -sensitive K^+ channel blocker glibenclamide

Table 3
mRNA expression levels of K_{ATP} channel subunits in the control and NAC groups calculated by the $\triangle\triangle C_T$ method.

Gene	Protein	Control	2 mM NAC	5 mM NAC	10 mM NAC
ABCC8	SUR1	1.48 ± 0.09	5.24 ± 0.791^{a}	$4{,}98 \pm 0.66^{a}$	5.80 ± 2.15^{a}
ABCC9	SUR2	2.02 ± 0.22	$1.12 \pm 0.125^{a,b,c}$	2.06 ± 0.083	1.71 ± 0.34
KCNJ8	Kir6.1	4.87 ± 0.30	1.58 ± 0.54^{a}	3.35 ± 1.48	2.58 ± 1.73
KCNJ11	Kir6.2	4.54 ± 3.93	4.53 ± 1.57	1.68 ± 0.73	2.58 ± 1.85

[a]When compared to the expression level in the control group, $p < 0.05$, [b]When compared to the expression level in the 5 mM NAC group, $p < 0.05$, [c]When compared to the expression level in the 10 mM NAC group, $p < 0.05$.

had no effect on the relaxation induced by NAC [9]. Additionally in the present study, we determined K_{ATP} channel density by using gene expression methods. ABCC8 expression was upregulated by NAC, whereas that of KCNJ11, KCNJ8 and ABCC9 remained unchanged following NAC treatment. We also found positive correlation between relaxation rate and mRNA expression level of ABCC8 subunit. ABCC8 encodes SUR1 receptor. These results, suggest that role of mRNA expression is partly in NAC induced relaxation. The most important features of K_{ATP} are rapid and reversible closure by cytoplasmic ATP, and activation by nucleotide tri-and diposphates. The K_{ATP} channels consist of four subunits (Kir 6.1 or Kir 6.2). Each subunit is associated with regulatory suphonylurea receptor (SUR). Channel activation then arises from the activating effects of other nucleotides on the SUR subunit [22]. Our results demonstrate that NAC activates aortic K_{ATP} channels. NAC may be activate directly these nucleotides.

In the present study, to show the vasodilatory effect of NAC, the isolated thoracic aorta rings were contracted with 60 mM KCl. After the contraction force reached a plateau value, 2, 5 or 10 mM NAC was added and relaxation responses were recorded. We found that NAC concentration-dependently increased the values of the relaxation and decreased intracellular calcium concentration compared to those at baseline. Additionally, we observed a significant positive correlation between relaxation rate and % decrease of intracellular calcium concentration. These findings are consistent with the results reported by Han et al. [9]. Adenosine, calcitonin gene-associated peptide, prostacyclin, and beta agonists cause hyperpolarization by increasing K_{ATP} channel activity in the vascular smooth muscles and induce vasodilatation by decreasing Ca^{2+} influx [23,24]. The increase in the K^+ channel conductance and decrease of the intracellular Ca^{2+} concentration caused by NAC in

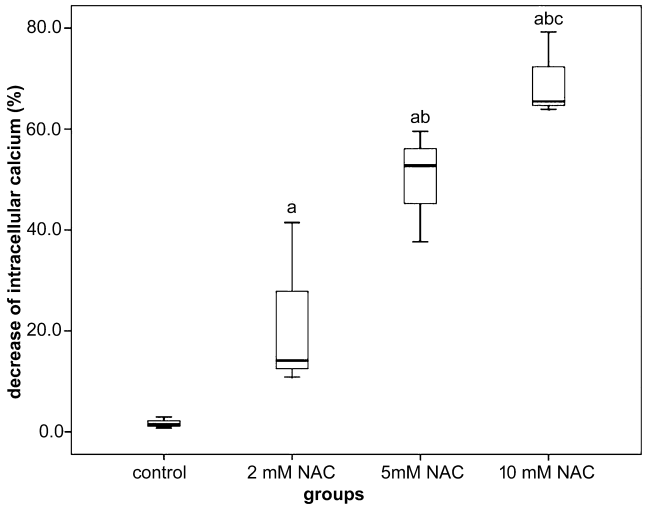

Fig. 7. Concentration-dependent decrease of the intracellular calcium concentration in NAC-treated cultured smooth muscle cells. [a]When compared to the value in the control group, $p < 0.05$; [b]When compared to the value in the 2 mM NAC group, $p < 0.05$; [c]When compared to the value in the 5 mM NAC group, $p < 0.05$.

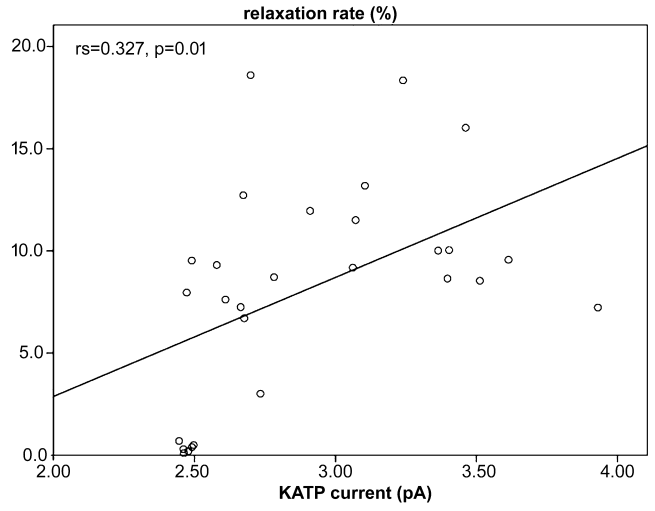

Fig. 8. Scatter plot of relaxation rate and K_{ATP} channel current. Pearson correlation analysis shows a significant positive correlation of K_{ATP} channel current versus relaxation rate (Pearson r = 0.477; $p < 0.05$). Line represents linear regression of data. rs = r square.

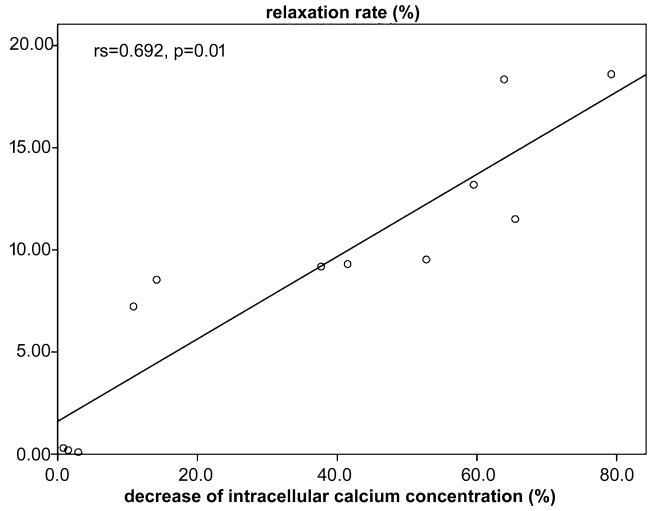

Fig. 9. Scatter plot of relaxation rate and decrease of intracellular calcium concentration. Pearson correlation analysis shows a significant positive correlation of decrease of intracellular calcium versus relaxation rate (Pearson r = 0.921; $p < 0.01$). Line represents linear regression of data. rs = r square.

our experiments indicate that NAC vasodilatory effect on the thoracic aorta smooth muscle cells is mediated *via* the modulation of K_{ATP} channels and intracellular calcium concentration, *i.e.*, similarly to the mechanisms of action of the above mentioned substances. Briefly, the increase in efflux of K^+ through K_{ATP} channel causes hyperpolarization of the membrane of thoracic aorta. This effect is followed by the closure of voltage-dependent L-type Ca^{2+} channels. As a result, calcium entry and intracellular calcium concentration decrease and vasodilation occurs.

NAC is a source of sulfhydryl groups in cells and scavenger of free radicals as it interacts with reactive oxygen species (ROS) such as $OH^{\bullet}$ and H_2O_2. Vasodilatation could also be achieved by NAC-mediated ROS scavenging which results in increased nitric oxide bioavailabilty and consequent attenuation of calcium entry [25]. The important limitation in our study, nitric oxide level is not determined.

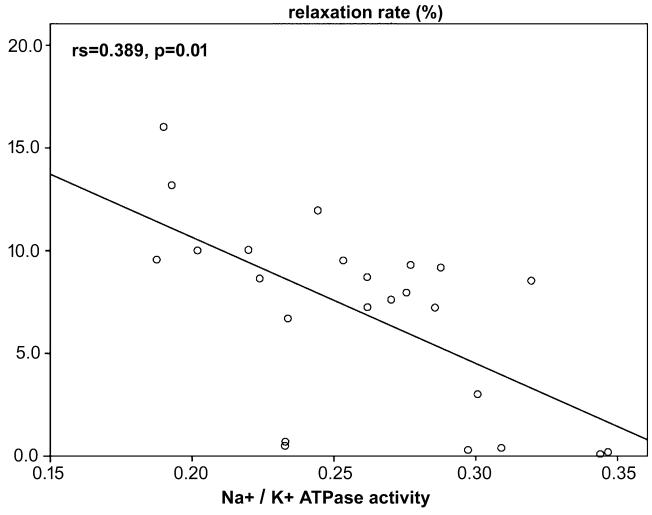

Fig. 10. Scatter plot of relaxation rate and Na^+/K^+-ATPase activity. Pearson correlation analysis shows a significant negative correlation of Na^+/K^+-ATPase activity versus relaxation rate (Pearson r = −0.624; $p < 0.01$). Line represents linear regression of data. rs = r square.

The Na^+K^+-ATPase activity is another important mechanism contributing to the maintenance of vascular tone and membrane potential of vascular smooth muscle cells [26]. In the present study, we also investigated the role of Na^+/K^+-ATPase by the NAC-induced vasodilatation and demonstrated that NAC inhibited Na^+/K^+-ATPase activity as partially. We observed negative correlation between Na^+/K^+-ATPase activity and relaxation rate. This was a surprise observation for us. According to our current knowledge, inhibition of Na^+/K^+-ATPase should cause depolarization of the membrane. But we have observed that the membrane was hyperpolarized and the vascular smooth muscle relaxed. This controversy may result from the fact that intracellular signal transduction mechanisms regulating Na^+K^+-ATPase activity depend on biochemical and biophysical features of the environment, such as oxygen, carbon dioxide, hormone and local tissue factors. Inhibition of the Na^+,K^+-ATPase activity, however, may change the ATP/ADP ratio and the transmembrane potassium gradient for the K_{ATP} channels. A similar relationship between the relaxation and the Na^+,K^+-ATPase activity has previously been shown by nitric oxide, a vasodilator agent, in the kidney [27] in liver [28], and in erythrocytes [29].

In conclusion, the role of K_{ATP} channels in NAC-induced vasodilatation was analyzed for the first time using electrophysiological and molecular techniques. We found that NAC-induced vasodilatation was modulated by K_{ATP} channel currents, intracellular Ca^{2+} concentrations and Na^+/K^+-ATPase activity. Our findings suggest that NAC may be used for the prevention of vasospasms in coronary bypass surgery and other vasospasm related cardiovascular diseases. However, further studies are required for the elucidation of the signaling pathways of Ca^{+2}, Na^+/K^+-ATPase and K_{ATP} in the NAC induced vasodilatation.

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