Comparison of the post-mortem interval on the effect of vascular responses to the activation of ionotropic and metabotropic receptors

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Abstract. The contractibility of blood vessels depends on their normal structure and the availability of calcium ions; it changes under the influence of numerous contracting and relaxing factors, which control the activities of various pathways of intracellular and intercellular signaling. The main aim of the study was to investigate, by means of perfusion pressure in rat tail arteries, the role of Ca^{2+} in vascular response to α-1 adrenoceptor activation by phenylephrine (PHE) and Bay K8644 agonist of the L-type calcium channel and caffeine before and after a post-mortem interval (PMI) of 2, 4, 6 and 8 h. A phasic increase of perfusion pressure in rat tail arteries, as induced by PHE or caffeine, in Ca^{2+}-free solutions was used as an indicator of intracellular Ca^{2+} release through the inositol 1,4,5-triphosphate and ryanodine receptor pathways, respectively. In Ca^{2+}-free-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) and in Ca^{2+}-EGTA-PSS, the PHE induced elevation of perfusion pressure significantly decreased. Vascular responses to caffeine (20 mmol/l) in Ca^{2+}-free-EGTA-PSS, with an increase of PMI from 2-8 h, did not change significantly. A similar effect was observed with vascular responses to KCl 40 mmol/l in Ca^{2+}-EGTA-PSS. To confirm whether the inhibitory effect of 2, 4, 6 and 8 h PMI was mediated through the formation of NO, nitro-L-arginine (L-NNA), a potent NO synthase inhibitor, was used. Exposure to L-NNA (10^{-5} M) blocked the inhibition induced by an increase of PMI. The blocked effects of L-NNA were reversed by L-arginine (10^{-4} M). In conclusion, these patterns of change in artery responses provide insight into the post-mortem change in the receptor-mediated signaling components in epithelial and smooth muscle cells, and support the further study of post-mortem vascular responses triggered by G protein-coupled receptors (metabotropic) and channel-linked receptors (ionotropic) as potential markers for estimating short and long-term PMIs, respectively.

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Introduction

Blood vessel contractibility depends on their normal structure and the availability of calcium ions. The contractibility changes under the influence of numerous contracting (such as angiotensin II and endothelin-1) and relaxing [including nitric oxide (NO) and prostacyclin] factors, which control the activities of various pathways of intracellular and intercellular signaling. Perfusion pressure in rat tail arteries were investigated in the present study, to investigate the role of Ca^{2+} in vascular response to activation α-1 adrenoceptor by phenylephrine (PHE) and Bay K8644 agonist of the L-type calcium channel and caffeine before and after a post-mortem interval (PMI) of 2, 4, 6 and 8 h. An elevated intracellular concentration of free calcium ions is considered a primary toxic mechanism in tissue damage (1-5). Mobilization tissues and an influx of calcium has long been considered as a major mechanism of ischemic cell death induced by different means, including ischemia (7-10). The increased concentration of cytoplasmic free-Ca^{2+} following hypoxia is derived from an influx and release from intracellular storage, such as endoplasmic reticulum (ER) and mitochondria.

Previous evidence indicated that mobilization of the intracellular calcium is in itself sufficient to activate apoptosis (10). This is supported by our finding that inhibition of nitric oxide synthase (NOS) elevated resistance to treatment with $CaCl_2$, which mimics hypoxic stress and leads to the influx of calcium (5,6). Treatment with 300 μM $CaCl_2$ is commonly used to mimic hypoxic insults *in vivo* and *in vitro*.

The decrease in calcium influx $(Ca^{2+})_i$ can be observed through different mechanisms, including inhibition of Ca^{2+} entry across the plasma membrane and inhibition of intracellular Ca^{2+} release. NO has an inhibitory effect on Ca^{2+} entry through the voltage-gated calcium channel in vascular smooth

muscle. Inconsistent results have been reported regarding how NO affects intracellular Ca^{2+} release. The vascular relaxation by NO is caused partly by the reduction of intracellular Ca^{2+} release. NO has been found to not influence arg^8-vasopressin-induced intracellular Ca^{2+} release (5).

The present study was designed to investigate whether NO interfered with the Ca^{2+} influx from the extracellular space and release from intracellular storage. L-NNA was used to analyze the role of NO in the modification of post mortem vascular reactivity. More over experiments on normal arteries and endothelium denudated arteries were performed to got clear results.

A phasic increase of perfusion pressure in rat tail arteries induced by PHE or caffeine in Ca^{2+}-free solution was used as an indicator of intracellular Ca^{2+} release through the inositol 1,4,5-triphosphate (IP_3) and ryanodine receptor pathways, respectively. In Ca^{2+}-free-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) in the presence Bay K8644 and KCl did not increase of perfusion pressure (11). Bay K and KCl induced an increase of perfusion pressure that was observed only in Ca^{2+}-PSS. The increases of perfusion pressure induced by Bay K and KCl were used as an indicator of Ca^{2+}-influx from extracellular space to cytoplasm through the Ca^{2+}-channel in the cellular membrane.

The influence of PMI on the PHE and caffeine treatment evoked a phasic increase of perfusion pressure in Ca^{2+}-free solution in endothelium intact and endothelium-denuded rat tail arteries were studied.

Materials and methods

Perfusion pressure study. A male Wistar rat (250-300 g) was anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally), and its tail artery was removed quickly and placed in cold Krebs' solution of the following composition (mmol/l): 115.0 NaCl, 5.0 KCl, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 2.1 $CaCl_2$ and 11.0 glucose. Fat and connective tissues surrounding the rat tail arteries were removed carefully and the isolated artery was cannulated at the two ends. The tail arteries were then suspended in organ chambers containing 20 ml of Krebs-Ringer bicarbonate solution containing (mmol/l) 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$ and 11.1 glucose (control solution) at 37˚C and bubbled through with 95% O_2 and 5% CO_2. One of the wires was anchored in the organ chamber and the other was connected to a pressure transducer Statham-Gould type P23ID connected with a polyphysiograph (Narco Bio System, Inc., Houston, TX, USA) Narcotrace 40 and digital recorder Graphtec midi Logger GL820 (Graphtec Corporation, Tokyo, Japan). The rat tail artery was equilibrated at an initial resting tension of 500 mg and the buffer solution was changed every 15 min to stabilize the arterial tissues. The endothelium was removed from specific arteries when necessary. The removal of the endothelium was verified functionally by the lack of relaxant response to acetylcholine (10^{-5} M) in rat tail arteries precontracted with PHE (10^{-6} M) (12,13). The study protocol was approved by the Local Ethics Committee and all experiments were carried out in accordance with the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication No. (NIH) 85-23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, USA]. The study used 15 animals. All animals were purchased from Trójmiejska Akademicka Zwierzętarnia Doświadczalna Centrum Badawczo Usługowe GUMed (Gdańsk, Poland).

Ca^{2+}-free-EGTA solution was prepared by the omission of $CaCl_2$ and the addition of 0.2 mmol/l EGTA. Prior to data collection, all the rat tails were contracted with PHE (10^{-6} M) or caffeine (20 mmol/l) in Ca^{2+}-free solution for 5 min to deplete the internal Ca^{2+} sources.

As the second application of the agonists was significantly diminished, the arterial tissues were subsequently washed with Ca^{2+}-containing Krebs solution three times and incubated for 15 min (Ca^{2+} loading time). Following this the medium was replaced by Ca^{2+}-free solution and 5 min later PHE (10^{-6} M) or caffeine (20 mmol/l) was applied to produce a phasic increase of perfusion pressure. Only those tissues exhibiting repeated perfusion pressure responses of a similar magnitude in Ca^{2+}-free solution were selected. In all the experiments, L-NNA, L-arginine, D-arginine, sodium nitroprusside (SNP; 10-7M) and 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; 10-5M) were added to the organ chambers at the beginning of the incubation period with Ca2+-free solution. SNP and ODQ were purchased from Sigma Aldrich (Poznan, Poland).

Data analysis. The results are presented as means ± standard error. Statistical analysis was performed using the Newman-Keuls test for multiple comparison of the means, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

PMI on perfusion pressure. The influence of PMI on perfusion pressure induced by PHE, caffeine, Bay K8644 and KCl was investigated in rat tail arteries in Ca^{2+}-EGTA-PSS, Ca^{2+}-free-EGTA-PSS, with and without endothelium.

PHE (10^{-6} M) and caffeine (20 mmol/l) evoked a rapid increase of the perfusion pressure of the rat tail arteries in Ca^{2+}-free solution, 137±12 and 73±12 mmHg, respectively (Fig. 1). Perfusion pressure reached its maximal levels within 40 sec and declined thereafter without a sustained perfusion pressure. To exclude the possibility that residual Ca^{2+} in Ca^{2+}-free solution may play a role in the contractile responses, KCl (40 mmol/l), which can only stimulate Ca^{2+} entry through the plasma membrane, was applied after incubation with Ca^{2+}-free solution for 5 min, but it could not induce an increase of perfusion pressure. Bay K8644 (10^{-6} M), which can stimulate only Ca^{2+} entry through the plasma membrane, was also applied after incubation with Ca^{2+}-free solution for 5 min, but it also could not induce an increase perfusion pressure. Thus, an increase of perfusion pressure induced in Ca^{2+}-free-EGTA-PSS by PHE and caffeine are caused by intracellular Ca^{2+}-release by two different mechanisms, IP_3-dependant and -independent, respectively.

In Ca^{2+}-EGTA-PSS solution, PHE and caffeine induced a significantly greater increase of perfusion pressure, which reached 183±11 mmHg (n=16) and 93±16 mmHg (n=14) respectively. Furthermore, KCl and Bay K8644 induced an increase of perfusion pressure in Ca^{2+}-EGTA-PSS only. A mean value of maximal perfusion pressure obtained with KCl

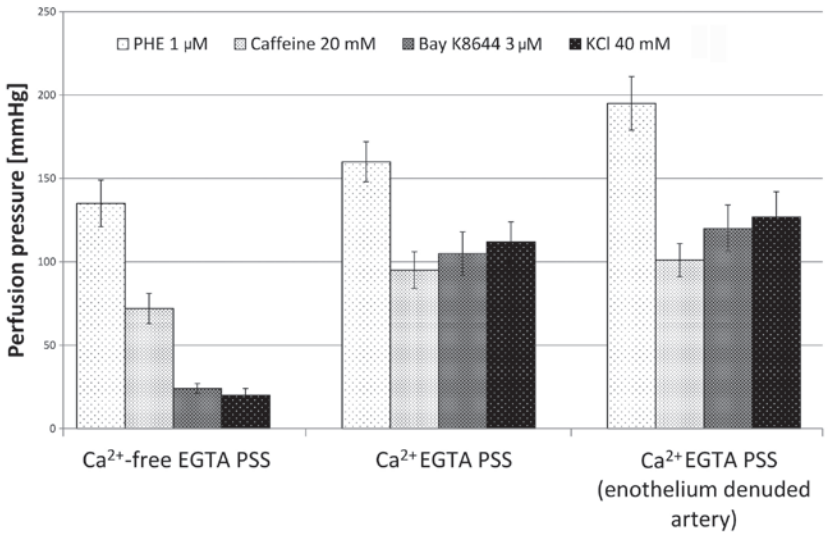

Figure 1. Response of perfused rat tail artery to 1 μM phenylephrine (PHE), 20 mmol/l caffeine, 3 μM Bay K8644 and 40 mmol/l KCl in the Ca^{2+}-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) and Ca^{2+}-free-EGTA-PSS for control and endothelium-denuded arteries. Data represent means ± standard error of the mean for four experiments.

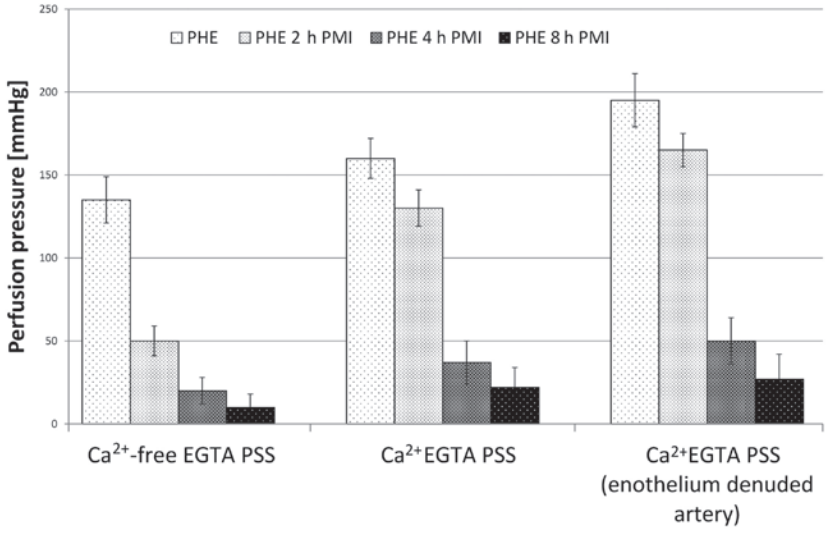

Figure 2. Influence of post-mortem interval (PMI) (2, 4 and 8 h) on the responses of perfused rat tail arteries to 1 μM phenylephrine (PHE) in the Ca^{2+}-EGTA-PSS and Ca^{2+}-free-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) for control and endothelium-denuded arteries. Data represent means ± standard error for n=16.

(40 mmol/l) and Bay K8644 reached 116±15 and 84±10 mmHg, respectively (Fig. 1).

In the Ca^{2+}-free-EGTA-PSS solution after an increase of PMI from 2-8 h, PHE induced elevation of perfusion pressure significantly decreased. After 2 h PMI, an increase of maximal perfusion pressure induced by PHE reached 49±14 mmHg only (n=16). Furthermore, an increase of PMI to 4 and 8 h depleted the response on PHE to 16±9 and 8±7 mmHg, respectively (Fig. 2). PHE also induced an increase of perfusion pressure in Ca^{2+}-EGTA-PSS, which significantly decreased with the increase of PMI. The mean maximal response to PHE reached 183±22 mmHg in the control and after 2, 4 and 8 h PMI depleted to 137±23, 34±12 and 18±9 mmHg, respectively (Fig. 2).

By contrast, rat tail artery responses to caffeine (20 mmol/l) in Ca^{2+}-free-EGTA-PSS solution did not change significantly with an increase of PMI from 2-8 h (Fig. 3). The mean maximal responses oscillated from 78±15 mmHg after 2 h PMI to 62±11 mmHg after 8 h PMI (Fig. 3).

Similar effects in Ca^{2+}-EGTA-PSS were observed with KCl (40 mmol/l), which induced an increase in perfusion pressure of the rat tail arteries. In this condition, the mean maximal responses slightly decreased and oscillated between 116±15 mmHg in the control response and 121±17, 119±14 and 98±12 mmHg after 2, 4 and 8 h PMI, respectively (Fig. 4).

Discussion

The estimation of PMI remains an ambiguous problem in forensic investigations (3). The use of calmodulin (CaM) binding proteins (CaMBPs) as indicators of PMI has been investigated previously. In lung tissue samples, Ca^{2+}/CaM-dependent protein

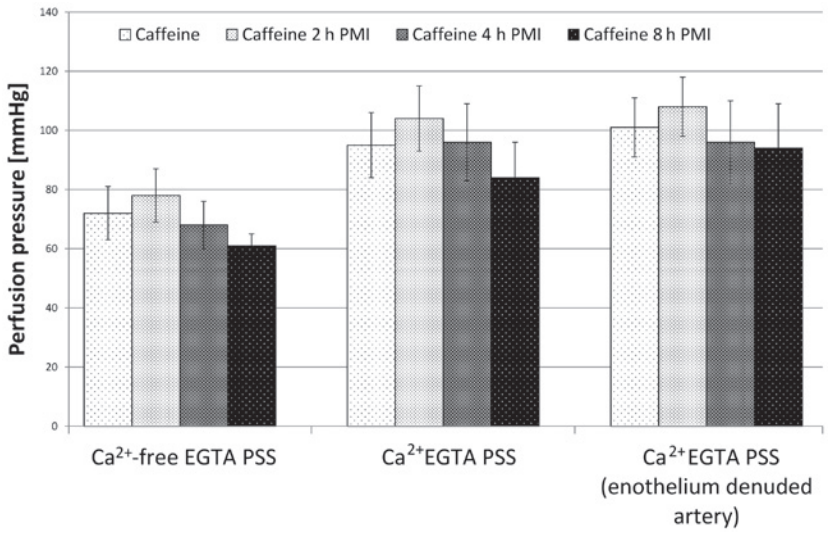

Figure 3. Influence of post-mortem interval (PMI) (2, 4 and 8 h) on the responses of perfused rat tail arteries to 20 mmol/l caffeine in the Ca^{2+}-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) and Ca^{2+}-free-EGTA-PSS for control and endothelium-denuded arteries. Data represents means ± standard error for n=12.

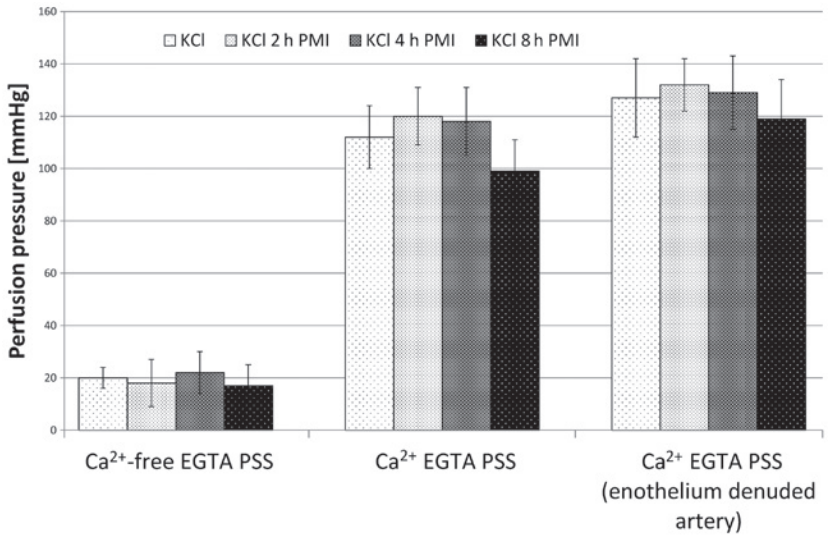

Figure 4. Influence of post-mortem interval (PMI) (2, 4 and 8 h) on the responses of perfused rat tail arteries to 40 mmol/l KCl, in the Ca^{2+}-ethylene glycol tetraacetic acid (EGTA)-poly(sodium styrenesulfonate) (PSS) and Ca^{2+}-free-EGTA-PSS for control and endothelium-denuded arteries. Data represents means ± standard error for n=14.

kinase II (CaMKII) levels did not significantly change over the 96 h PMI time period examined. The stability of CaMKII levels over 96 h was also observed in skeletal muscle tissue, whereas calcineurin A (CNA) showed variable levels at 0, 48 and 96 h with the presence of the rapidly migrating band at 24 h. Kang *et al* analyzed specific CaMBPs, Ca(2+)/CaM-dependent kinase II, calcineurin A in immunoblot analysis. These patterns of change in CaMBPs provide insight into the post-mortem changes in CaM-mediated signaling components in lung and skeletal muscle and support the further study of CNA and CaMKII as potential markers for estimating short- and long-term PMIs (3).

By contrast, inducible NOS levels, which vary between samples, CNA and myristoylated alanine-rich C-kinase substrate (MARCKS) exhibited predictable patterns of change; the level of MARCKS decreased steadily in the 0-96 h post-mortem lung samples (14).

In the present study, the possible use of vascular responses to PHE, Bay K, caffeine and KCl were examined as indicators of PMI. All the models were allowed to determine the time of laying within a period of ~2 h and consequently this was determined as the time of death; under favorable conditions the species lays immediately following death.

The majority of systemic vessels relax, but pulmonary vessels contract when exposed to hypoxia; the mechanisms are likely to be diverse. Vascular and cardiac endothelium releases are the last three chemical messengers that may influence vascular and cardiac performance. First, endothelial cells express NOS (constitutive and inducible form). NO release by endothelial cells has been shown to elevate vascular cGMP. Post-mortem, the energy metabolism is disrupted and the endothelial cells lose Ca^{2+} from the ER and the cytosolic Ca^{2+} concentration increases. An increase in Ca^{2+} may also occur under pathophysiological

conditions, such as during hypoxia or ischemia, when the endothelial cells start developing an energy deficit. The study by Coburn *et al* (15) indicated that hypoxic relaxation is not only a function of energy stores, and that oxidative metabolism-contraction coupling is regulated by energy delivery to a reaction, or reactions that are controlling muscle force.

Tosun *et al* (9) suggested that oxygen sensing mechanisms may modulate the cell signaling pathways involved in excitation-contraction coupling, including mechanisms in which Ca^{2+} is the regulated parameter, for example by oxygen-dependent or adenosine triphosphate-dependent changes in Ca^{2+} permeability.

The present study investigated, by means of perfusion pressure in rat tail arteries, the role of the Ca^{2+} in vascular response to activation of α-1 adrenoceptor by PHE and Bay K8644 (agonist of L-type calcium channel) before and after 2, 4, 6 and 8 h PMI. A phasic increase of perfusion pressure in the rat tail arteries induced by PHE or caffeine in Ca^{2+}-free solution were used as an indicator of intracellular Ca^{2+} release through the inositol IP_3 and ryanodine receptor pathways, respectively. Bay K8644 and KCl induced an increase in perfusion pressure in Ca^{2+}-PSS only, and this increase was used as an indicator of Ca^{2+}-influx from the extracellular space to the cytoplasm through the Ca^{2+}-channel in the cellular membrane. The influence of PMI on the PHE- and caffeine-induced phasic contractions in Ca^{2+}-free solution in the intact endothelium and endothelium denuded rat tail arteries was studied.

To confirm whether the inhibitory effect of 2, 4, 6 and 8 h PMI was mediated through NO formation, L-NNA was used and compared to the effect of the inhibitor, COX indomethacin. Exposure to L-NNA (10^{-5} M) blocked the inhibition induced by increase PMI. The blocked effects of L-NNA could be reversed by L-arginine (10^{-4} M). An elevated intracellular concentration of free calcium ions is considered a primary toxic mechanism in tissue damage (2,16-18). Mobilization tissues and the influx of calcium has long been considered as a major mechanism of ischemic cell death induced by different means, including ischemia (7,19-21). The increased concentration of cytoplasmic free Ca^{2+} following hypoxia is derived from an influx and release from intracellular storage, such as ER and mitochondria (6,8,22).

Previous evidence indicated that mobilization of the intracellular calcium is in itself sufficient to activate apoptosis (14). This is supported by the finding that NOS inhibition elevated the resistance to treatment with $CaCl_2$, which mimics hypoxic stress and leads to the influx of calcium (4,6,22). Treatment with 300 μM $CaCl_2$ is commonly used to mimic hypoxic insults *in vivo* and *in vitro*.

In conclusion, these patterns of change in arteries responses provide insight into the post-mortem change in receptor mediated signaling components in epithelial and smooth muscle cells and support the further study of post-mortem vascular responses triggered by G protein coupled receptors (metabotropic) and channel linked receptors (ionotropic) as potential markers for estimating short- and long-term PMIs, respectively.

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