jected into a systemic vein to identify the area at risk and the nonischemic area.[21] The heart was then immediately removed and sliced into serial transverse sections that were 6 to 7 mm in width. The nonischemic area was defined as the tissue showing blue staining. The ischemic region was harvested and incubated at 37°C for 20 to 30 minutes in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemical Company) in 0.1 mol/L phosphate buffer adjusted to pH 7.4. TTC stains the noninfarcted myocardium a brick-red color, indicating the presence of a formazan product created through the reduction of TTC by dehydrogenases in viable tissues. The infarct size was expressed as a percentage of the area at risk.

## Selection Criteria

To ensure that all of the animals included in analysis of infarct size were healthy and exposed to a similar extent of ischemia, the following criteria were used to exclude unsatisfactory dogs: (1) subendocardial collateral flow > 15 mL 100 $g^{-1}$ $min^{-1}$, (2) heart rate >170 bpm, and (3) more than 2 consecutive attempts required to convert ventricular fibrillation with low-energy DC pulses applied directly to the heart.

## Statistical Analysis

Statistical analysis was performed with ANOVA for comparisons among the groups. If ANOVA indicated a significant difference, paired data were compared using Bonferroni test.[22,23] Changes of the hemodynamic parameters over time were compared by ANOVA with repeated measures. Using endocardial collateral blood flow in the inner half of the LV wall as the covariate, ANCOVA was performed to assess the influence of collateral flow on infarct size. Results are expressed as the mean ± SEM, and $P < 0.05$ was considered significant.

## RESULTS

Seventy dogs were randomly assigned to 9 different protocols and the infarct size was determined in each group. Eight dogs were excluded from analysis because subendocardial collateral flow was greater than 15 mL 100 $g^{-1}$ $min^{-1}$. Among the remaining 62 dogs, 17 developed ventricular fibrillation at least once and fibrillation that fulfilled the exclusion criteria occurred in 9 dogs, which were also excluded from the study.

Mean aortic blood pressure and heart rate (Fig. 1) did not vary among the 9 groups throughout the study. The percent area at risk in the left ventricle and the endocardial collateral blood flow during myocardial ischemia were also not significantly different among the 9 groups (Fig. 2). Figure 3 shows that MX-68 markedly reduced the infarct size compared with that in the control group. This effect of MX-68 was completely

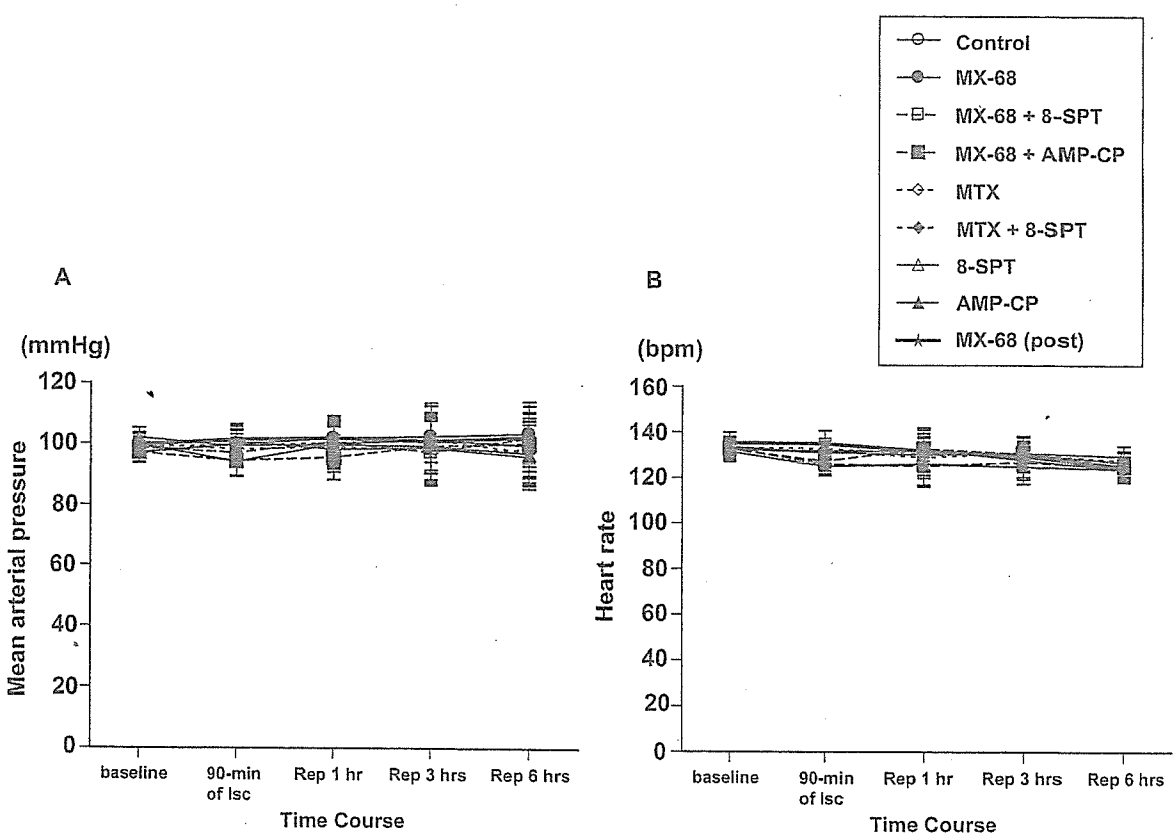


FIGURE 1. Systemic hemodynamic parameters (mean arterial pressure (A) and heart rate (B)) throughout the study. There were no significant changes of these parameters in all the 9 groups. Statistical significance was tested by ANOVA.

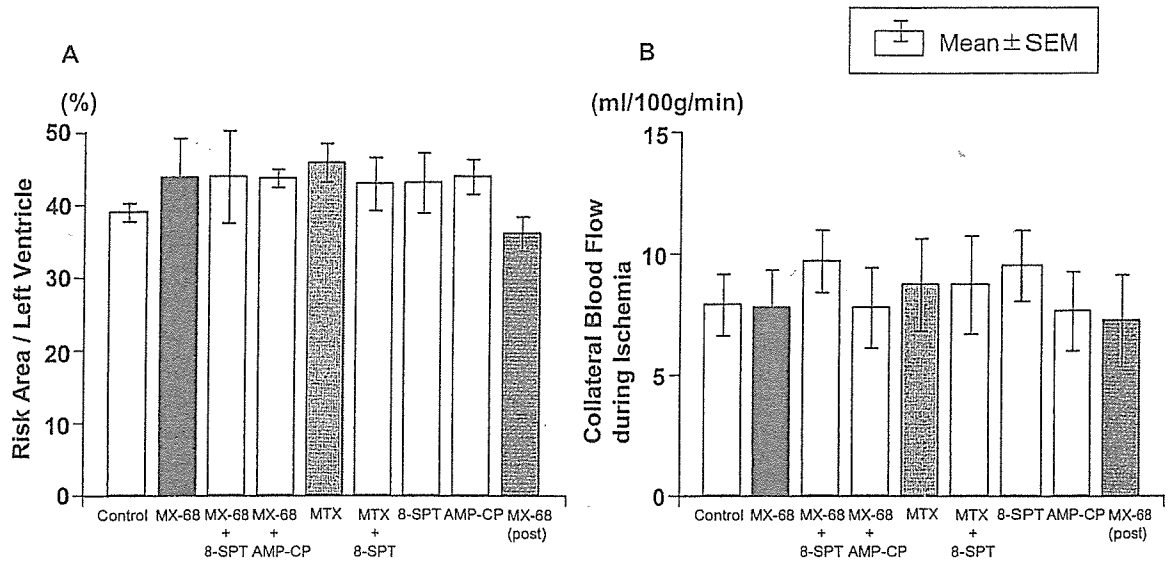


FIGURE 2. Area at risk (A) and collateral blood flow during ischemia (B) in the control group, MTX group, MX-68 group, MTX+8-SPT group, MX-68+8-SPT group, MX-68+AMP-CP group, MX-68(post) group, 8-SPT group, and AMP-CP group. There were no differences of the area at risk and collateral flow during ischemia between these groups. Statistical significance was tested by ANOVA.

blunted by infusion of either 8-SPT or AMP-CP. MTX also reduced infarct size in a similar manner to MX-68, and its protective effect was blunted by 8-SPT. Even when MX-68 was administered after the start of reperfusion, reduction of infarct size was observed to a level between that in the control group and that when MX-68 was administered before ischemia. Regression plots of infarct size versus collateral blood flow are shown in Figure 4, which indicate that the infarct size-limiting effect of either MX-68 or MTX was attributable to an adenosine-dependent mechanism.

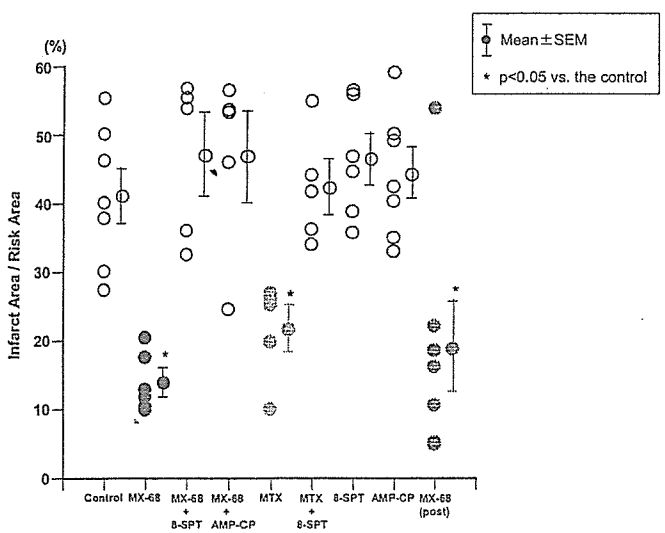


FIGURE 3. Infarct size expressed as a percentage of the area at risk. Infarct size was markedly decreased in the MTX and MX-68 groups compared with the control group, and this improvement was completely reversed by 8-SPT or AMP-CP. Statistical significance was tested by ANOVA.

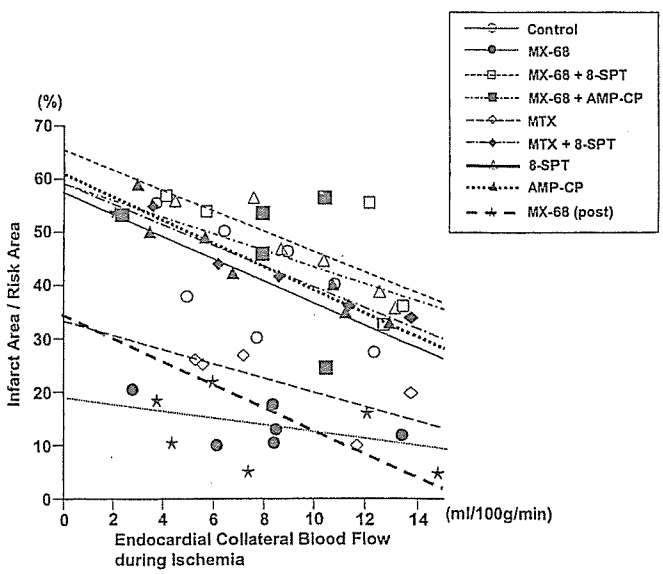


FIGURE 4. Infarct size after 90 minutes of ischemia versus regional collateral flow during ischemia. Infarct size is expressed as a percentage of the area at risk. Infarct size was markedly decreased in the MTX and MX-68 groups compared with the control group. This improvement was completely reversed by 8-SPT or AMP-CP. Statistical significance was tested by ANCOVA.

## DISCUSSION

The present study demonstrated that either MTX or MX-68 could markedly reduce infarct size and that the cardioprotective effects of these agents were attributable to ecto-5′-nucleotidase- and adenosine-dependent mechanisms.

### Adenosine and the Cardioprotective Effect of MTX or MX-68

Anti-inflammatory drugs such as steroids were thought to have an infarct size-limiting effect[7,8] because the pathophysiology of myocardial infarction resembles tissue inflammation and such drugs can potently ameliorate the tissue inflammatory process. However, steroids and related hormones have been variously reported to decrease infarct size, have no effect, or even increase infarct size.[4,9] On the other hand, other anti-inflammatory agents seem to be effective against ischemia/reperfusion injury. For example, 17β-estradiol is known to have an anti-inflammatory effect[10] and it markedly reduces infarct size.[11] Statins are known to improve vascular inflammation and atherosclerosis, and these drugs also reduce infarct size markedly.[12] Therefore, we cannot necessarily conclude that all anti-inflammatory drugs will be effective for ischemia/reperfusion injury, but we can suggest that these drugs have the possibility of mediating cardioprotection.

MTX and its analog MX-68 are disease-modifying antirheumatic drugs,[20] and their mechanism of action on immune cells was recently reported to be mediated via adenosine.[13] If this is the case in myocardial cells, either MTX or MX-68 would limit infarct size because adenosine markedly reduces the size of infarcts and triggers/mediates the cardioprotective effect of ischemic preconditioning.[24,25] Indeed, the present study revealed that MTX and its analog (MX-68) can ameliorate ischemia/reperfusion injury. We also showed that this action is adenosine-dependent, because the effect of either MTX or MX-68 was blunted by 8-SPT, an adenosine receptor antagonist. Accordingly, both MTX and MX-68 ameliorate ischemia/reperfusion injury via adenosine-related mechanisms.

In immune system cells, the adenosine-related action of MTX was reported to be attributable to ecto-5′-nucleotidase,[13] and this also seems to be the case in the myocardium because the effect of MX-68 and MTX was blunted by an ecto-5′-nucleotidase inhibitor or an adenosine receptor antagonist. Ecto-5′-nucleotidase produces adenosine, and adenosine inhibits norepinephrine release from presynaptic vesicles and attenuates $Ca^{2+}$ influx into myocytes by acting on $A_1$ receptors and inhibitory G protein.[26,27] Adenosine also increases CBF, inhibits platelet aggregation, and inhibits leukocyte activation via $A_2$ receptors and stimulatory G protein.[14,16] Since factors such as an increase of norepinephrine, $Ca^{2+}$ overload, decreased CBF, and activation of platelets and leukocytes are deleterious to the heart, control of these factors by adenosine may help to minimize ischemia/reperfusion injury. Several studies have shown that adenosine administration markedly attenuates ischemia/reperfusion injury.[3,15,17]

### Role of Adenosine in the Effect of MTX or MX-68

How does MTX or MX-68 act on ecto-5′-nucleotidase? Several possibilities can be suggested. First, activation of ecto-5′-nucleotidase may occur after phosphorylation, as seen with ischemic preconditioning or treatment with phorbol ester, where activation of protein kinase C possibly leads to the phosphorylation and activation of ecto-5′-nucleotidase.[28,29] However, the in vitro activity of myocardial ecto-5′-nucleotidase was not increased by brief exposure to MTX (data not shown), whereas methoxamine and phorbol ester, which phosphorylate and activate ecto-5′-nucleotidase, both activated myocardial ecto-5′-nucleotidase in vitro.[28,29] These results suggest that MTX does not activate ecto-5′-nucleotidase via the process of phosphorylation, so a direct interaction between MTX and the active site of ecto-5′- nucleotidase may be responsible instead.

Second, MTX is reported to increase the tissue level of AICA riboside by inhibition of AICA riboside deaminase,[13] and we have previously shown that AICA riboside increases the activity of ecto-5′-nucleotidase.[19] Therefore, ecto-5′-nucleotidase may be activated when the myocardial AICA riboside is increased during administration of MTX in vivo. However, it has not been clarified how AICA riboside activates ecto-5′-nucleotidase in the heart. Since AICA riboside activates AMP deaminase and inactivates adenosine deaminase, it may also modulate the enzymes related to adenosine metabolism.[30] Accordingly, AICA riboside could increase adenosine production via activation of ecto-5′-nucleotidase, and maintain a high adenosine level by inhibiting enzymes involved in the metabolism of adenosine. In this context, there are many reports that AICA riboside is cardioprotective against ischemia/reperfusion injury via adenosine-dependent mechanisms.[16,30,31]

### Clinical Relevance and Limitations

In this study, we demonstrated that both MX-68 and MTX can limit infarct size via adenosine-dependent mechanisms. It would be of interest to test the cardioprotective effect of MTX or MX-68 in the clinical setting of acute myocardial infarction with coronary revascularization, since infusion of adenosine during reperfusion has been shown to limit infarct size.[17] Furthermore, since administration of adenosine can precondition the myocardium prior to sustained ischemia,[24] treatment with MTX or MX-68 may be useful in patients who have coronary artery disease to precondition the myocardium and improve resistance to acute myocardial infarction. However, further studies are necessary to develop either MTX or MX-68 as a drug to treat acute ischemic heart disease.

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Short communication

# In vivo assessment of catechol *O*-methyltransferase activity in rabbit skeletal muscle

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**Abstract**

With the use of microdialysis technique in the anesthetized rabbit, we examined the catechol *O*-methyltransferase (COMT) activity at the skeletal muscle interstitium. We implanted a dialysis probe into the adductor muscle, and monitored dialysate catecholamines and their metabolites with chromatogram-electrochemical detection. Administration of COMT inhibitor (entacapone) decreased dialysate 3-methoxy 4-hydroxyphenylglycol (MHPG) levels. Local administration of dihydroxyphenylglycol induced increases in dialysate MHPG levels. These increases in dialysate MHPG levels were suppressed by the addition of entacapone. The concentration of MHPG in the skeletal muscle dialysate corresponded to the COMT activity in the skeletal muscle. Furthermore, local administration of norepinephrine or epinephrine increased normetanephrine or metanephrine levels in dialysate but not MHPG levels. Skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.


*Keywords:* Catecholamine; Catechol *O*-methyltransferase; Entacapone; Microdialysis; Skeletal muscle

Catechol *O*-methyltransferase (COMT) exerts a critical action on the inactivation of catecholamrines and catecholestrogens (Boulton and Eisenhofer, 1998). COMT enzyme exists in almost all mammalian tissues and organs (Karhunen et al., 1994; Männistö and Kaakkola, 1999). The wide distribution of COMT in different tissues suggests an important physiological role for COMT activity. In vitro COMT activity has been widely assessed in various tissues (Männistö and Kaakkola, 1999; Tsunoda et al., 2002), while in vivo COMT activity has been assessed only in erythrocyte (Toumainen et al., 1996). To determine whether COMT activity is involved in cardiovascular regulation, we need information about in vivo COMT activity in organs and tissues.

A sophisticated technique using radiotracers has been employed for spillover of organ specific metabolite formed by COMT activity (3-methoxy 4-hydroxyphenylglycol, MHPG) (Lambert et al., 1995). This study suggested that majority of MHPG in plasma was derived from skeletal muscle, with the exception of central nervous system. Dispersed organs, such as skeletal muscle, have a thin and diffuse sympathetic innervation, but skeletal muscle is one candidate suitable for investigating regional MHPG production (Tokunaga et al., 2003a,b). This organ is suited to microdialysis probe implantation. Recently we have developed the skeletal muscle microdialysis for the monitoring of catecholamines and their metabolites. At the skeletal muscle, the small amounts of dialysate norepinephrine and its metabolites could be determined by microdialysis with electrochemical detection.

In the present study, we examined whether COMT blocker affected regional norepinephrine kinetics at the skeletal muscle interstitial spaces. With the use of dialysis technique, the dialysate was sampled from the skeletal muscle, and dialysate catecholamines and their metabolites levels were measured with liquid chromatography. Further, the study was designed to examine regional *O*-methylation products evoked by local administration of catecholamine and determine whether these data provide information about in vivo regional COMT activity.

Male Japanese white rabbits weighing 2.6–3.1 kg each were anesthetized with pentobarbital sodium (30–35 mg/kg,

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i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). After tracheotomy, the animals were ventilated with room air mixed with oxygen. Body temperature was maintained with a heated pad and lamp. All protocols were performed in accordance with the American Physiological Society guidelines for the use of animals. After a longitudinal skin incision was made in the left groin, the dialysis probes were implanted in the left adductor muscle with a fine guiding needle.

For skeletal muscle dialysis, we designed a transverse dialysis probe. The dialysis fiber (13 mm length, 0.31 mm O.D. and 0.2 mm I.D.; PAN-1200, 50,000 molecular mass cutoff, Asahi Chemical, Tokyo, Japan) was glued at both ends into a polyethylene tube (25 cm length, 0.5 mm O.D. and 0.2 mm I.D.) (Akiyama et al., 1991; Tokunaga et al., 2003a,b). The dialysis probe was perfused with Ringer solution at a speed of 10 μl/min using a microinjection pump (CMA 102, Carnegie Medicin, Stockholm, Sweden). Dialysate catecholamines and their metabolite concentrations were measured by high-performance liquid chromatography with electrochemical detection (Takauchi et al., 1997; Tokunaga et al., 2003a,b; Yamazaki et al., 1995).

Basal dialysate norepinephrine, dihydroxyphenylglycol (DHPG) and MHPG levels were presented in Table 1. Entacapone (COMT blocker) was intraperitoneally administered (10 mg/kg) (Illi et al., 1995; Scheinin et al., 1998). Administration of entacapone decreased the MHPG level of dialysate but increased the DHPG levels of dialysate. The dialysate norepinephrine levels were not affected by entacapone. These changes were preserved 2 h after administration of entacapone.

To examine regional COMT activity, we measured the formation of MHPG evoked by local administration of exogenous DHPG via dialysis probe. We determined doses of DHPG based on the dialysate DHPG concentration in the previous experiments (Akiyama and Yamazaki, 2001). Local administration of DHPG (25, 250 ng/ml) dose-dependently increased the MHPG levels of dialysate (Fig. 1). These increases in the MHPG levels were prevented by pretreatment with entacapone.

In this study, exogenous DHPG dose-dependently increased the MHPG levels of dialysate. Exogenous DHPG via the dialysis probe easily traversed the cell membrane and reached skeletal muscle (Goldstein et al., 1998). In contrast, entacapone significantly decreased the MHPG levels of

Table 1
Basal dialysate NE, DHPG, and MHPG levels in rabbit skeletal muscle

| | Before entacapone | After entacapone |
|---|---|---|
| NE (pg/ml) | 8±1 | 10±1 |
| DHPG (pg/ml) | 27±4 | 53±11* |
| MHPG (pg/ml) | 198±12 | 147±18* |

NE, norepinephrine; DHPG, dihydroxyphenylglycol; MHPG, 3-methoxy 4-hydroxyphenylglycol. Values are means±SE. $n$=5.
* $P$<0.05 vs. values before entacapone.

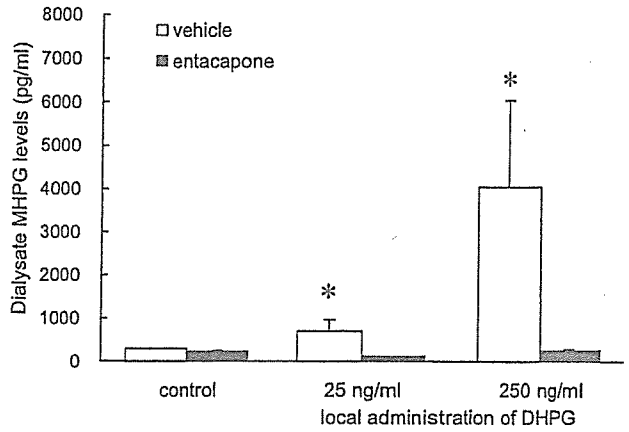


Fig. 1. Effects of exogenous dihydroxyphenylglycol (DHPG) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of DHPG dose-dependently increased the MHPG levels of dialysate. These increases in the MHPG levels were prevented by pretreatment with entacapone. Values are means±SE ($n$=5). *$P$<0.05 vs. control.

dialysate. These data suggest that regional COMT activity corresponds to the production of dialysate MHPG levels. Furthermore, entacapone induced a decrease in the dialysate MHPG level accompanied by an increase in the dialysate DHPG but not norepinephrine level. Therefore we consider that regional DHPG is one possible substrate for MHPG production, and that the concentration of MHPG or MHPG/DHPG ratio in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle.

Earlier studies suggested species and organ differences in extraneuronal uptake and COMT activity (Scheinin et al., 1998; Tsunoda et al., 2002). Extraneuronal norepinephrine uptake and COMT activity were well examined in rabbit heart with the findings suggesting that rabbit heart hardly metabolizes isoprenaline to methoxyprenaline (Lindmar and Löffelholz, 1974). Thus rabbit heart seems to have a very poorly developed extraneuronal system, including weak COMT activity, for the uptake and metabolism of catecholamines (Trendelenburg, 1978). On the other hand, rabbit aortic strips have a high capacity for COMT activity (Levin, 1974). From these and previous data (Tokunaga et al., 2003a,b), the ratio of MHPG/DHPG in myocardium and skeletal muscle were 1.0±0.2 and 7.9±1.3, respectively. Rabbit skeletal muscle seems to have a well-developed COMT activity. In the skeletal muscle sympathetic innervation was not dense, and the DHPG levels were less than that of heart (Tokunaga et al., 2003a,b). Therefore, other compounds or plasma DHPG might be involved in the regional formation of MHPG in the skeletal muscle.

MHPG is produced by extraneuronal *O*-methylation of DHPG formed intraneuronally from norepinephrine or by the extraneuronal combination of COMT and monoamine oxidase (MAO) on norepinephrine and epinephrine (Akiyama and Yamazaki, 2001; Eisenhofer et al., 1988). Therefore, MHPG is mainly yielded from DHPG, norepinephrine or epinephrine at the skeletal muscle. Furthermore,

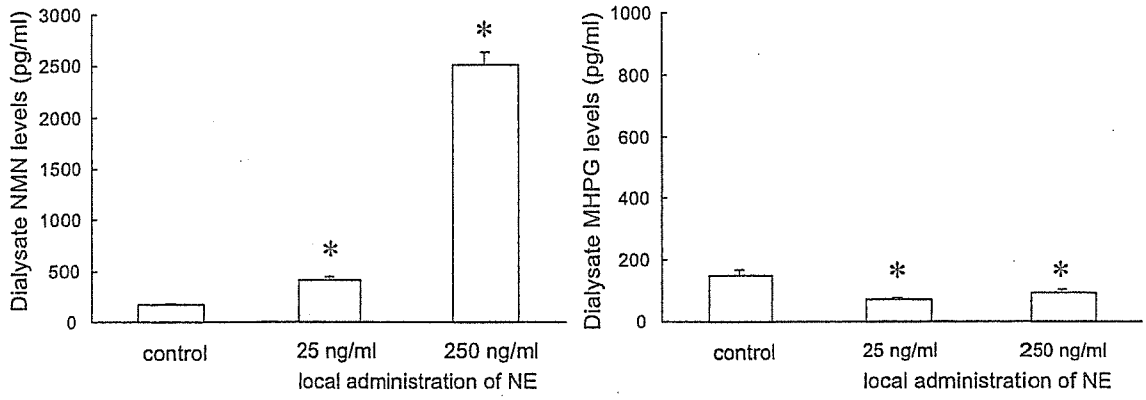


Fig. 2. Effects of exogenous norepinephrine (NE) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) and normetanephrine (NMN) production. Local administration of NE dose-dependently increased the NMN levels of dialysate but not MHPG levels. Values are means±SE ($n$=5). *$P$<0.05 vs. control.

*O*-methylation of catechol compounds includes MHPG, normetanephrine and metanephrine. We examined the relation between catecholamines and their metabolites. To compare norepinephrine and epinephrine with DHPG infusion, norepinephrine or epinephrine infusion with similar doses of DHPG was administered. Local administration of norepinephrine increased the normetanephrine levels of dialysate but not the MHPG levels (Fig. 2). Local administration of epinephrine increased the metanephrine levels of dialysate but not the MHPG levels (Fig. 3). Our data suggest that only DHPG is a possible substrate for MHPG production. Local administration of norepinephrine or epinephrine produced normetanephrine or metanephrine but not MHPG. Or rather, norepinephrine or epinephrine caused a decrease in the dialysate MHPG level. These data are consistent with data on the origins of plasma MHPG in rats, which indicated that most MHPG arises from *O*-methylation of the DHPG by intraneuronal deamination of norepinephrine (Eisenhofer et al., 1994).

Our data indicate that COMT exerts an important role on the degradation of cathecholamines in the skeletal muscular interstitium. Muscular catecholamines derive from circulating blood and surrounding sympathetic nerve systems (Tokunaga et al., 2003a,b). Therefore, COMT activity in the skeletal muscle may be related to regional or systemic sympathetic nerve activity. The relationship between regional COMT activity and sympathetic nerve activity remains to be further examined. Muscle sympathetic nerve activity is involved in the regulation of vascular tone and glucose metabolism in the skeletal muscle (Lundvall and Edfeldt, 1994; Spraul et al., 1994). Further studies concerning the physiological role of regional COMT activity on vascular or metabolic control are warranted.

To our knowledge, this is the first report on the in vivo assessment of COMT activity by direct measurement of dialysate MHPG, normetanephrine, and metanephrine obtained from skeletal muscle. Local administration of DHPG increased the MHPG levels of dialysate. These increases in MHPG were prevented by pretreatment with a COMT inhibitor. Therefore we consider that the concentration of MHPG in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle. Measurement of MHPG/DHPG ratio or MHPG formation evoked by DHPG infusion in skeletal muscle may be particularly appropriate for providing information about regional COMT activity. Thus skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

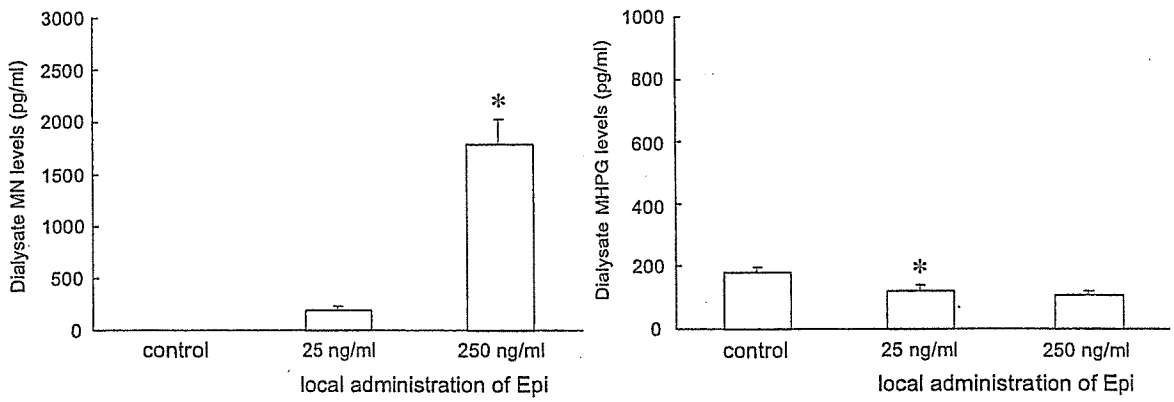


Fig. 3. Effects of exogenous epinephrine (Epi) infusion on the metanephrine (MN) and 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of Epi increased the MN levels of dialysate but not MHPG levels. Values are means±SE ($n$=5). *$P$<0.05 vs. control.

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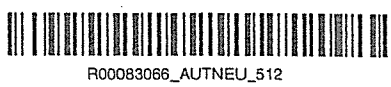

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# Extraneuronal enzymatic degradation of myocardial interstitial norepinephrine in the ischemic region

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**Abstract**

**Objective:** Catechol *O*-methyltransferase (COMT) is believed to exert degradative action at high norepinephrine (NE) levels. Although COMT exists in cardiac tissues, the contribution of cardiac COMT activity to regional NE kinetics, particularly in ischemia-induced NE accumulation, remains unclear. We investigated the role of cardiac COMT in NE kinetics in the ischemic region. **Methods:** We implanted a microdialysis probe into the left ventricular myocardium of anesthetized rabbits and induced myocardial ischemia by 60-min coronary artery occlusion. We monitored myocardial interstitial levels of NE and its metabolites in the presence and absence of a COMT inhibitor. We intraperitoneally administered entacapone (10 mg/kg) 120 min before control sampling. **Results:** In control, entacapone increased interstitial dihydroxyphenylglycol (DHPG, intraneuronal NE metabolite by monoamine oxidase (MAO)) levels and decreased interstitial normetanephrine (NMN, extraneuronal NE metabolite by COMT) and 3-methoxy-4-hydroxyphenylglycol (MHPG, extraneuronal DHPG metabolite by COMT) levels, but did not change interstitial NE levels. Coronary occlusion increased NE levels to 165 ± 48 nM at 45–60 min of occlusion. This increase was accompanied by increases in DHPG and NMN levels (11.3 ± 1.1 and 9.3 ± 1.3 nM at 45–60 min of occlusion). Entacapone augmented the ischemia-induced NE and DHPG responses (333 ± 51 and 22.9 ± 2.4 nM at 45–60 min of occlusion). In contrast, the ischemia-induced NMN response was suppressed by entacapone (2.0 ± 0.4 nM at 45–60 min of occlusion). Reperfusion decreased interstitial NE levels and increased interstitial DHPG and NMN levels. Entacapone suppressed changes in NE and NMN levels, but augmented the increase in dialysate DHPG. **Conclusion:** Myocardial ischemia evoked increases in myocardial interstitial NE and NMN levels. COMT inhibition augmented the increase in NE (substrate of COMT) levels and suppressed the increase in NMN (metabolite by COMT) levels. In the ischemic heart, COMT contributes to the removal of accumulated NE in the myocardium.


*Keywords:* Adrenergic agonists; Autonomic nervous system; Ischemia; Reperfusion; Neurotransmitters

## 1. Introduction

It has been reported that myocardial ischemia evokes an excessive norepinephrine (NE) accumulation in the myocardial interstitial space [1,2]. Outward NE transport through the uptake$_1$ carrier has been proposed as an important mechanism responsible for this ischemia-induced NE accumulation [2–4]. The presence of such high NE levels in the myocardial interstitium may be involved in the progression of myocardial cell injury and a higher incidence of malignant arrhythmia [5,6].

In the non-ischemic heart, released NE is reclaimed by cardiac sympathetic nerve endings via the uptake$_1$ carrier and repackaged or metabolized to dihydroxyphenylglycol (DHPG) by monoamine oxidase (MAO). NE, which escapes the synapses to the myocardial interstitium, spills over into the bloodstream or is taken up by extraneuronal cells via the uptake$_2$ carrier and mainly degraded to NE metabolites by catechol *O*-methyltransferase (COMT) [6–9] (Fig. 1). In the ischemic heart, normal transport by the uptake$_1$ carrier is impaired and NE spills over into the bloodstream, which is decreased due to the reduction of myocardial blood flow [1]. Therefore, extraneuronal enzymatic degradation may be the only mechanism that decreases myocardial interstitial NE. Little information, however, is available on the extraneuronal NE degradation by COMT in the ischemic region [10,11].

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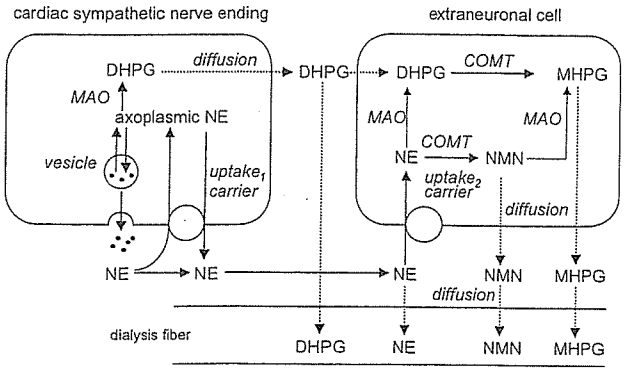


Fig. 1. Schema of putative factors affecting norepinephrine (NE) degradation in the cardiac sympathetic nerve ending and the extraneuronal cell. COMT, catechol *O*-methyltransferase; DHPG, dihydroxyphenylglycol; MAO, monoamine oxidase; NMN, normetanephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol.

Until now, available methodology for examination of organ-specific NE degradation has been limited to the assessment of radiolabelled-catecholamine kinetics [12]. Previously, using the dialysis technique in the in vivo heart, we demonstrated that coronary occlusion evokes a marked increase of myocardial interstitial NE levels in the ischemic region [1,2,4] and that outward NE transport through the uptake$_1$ carrier is involved in this NE efflux [2–4]. Moreover, we recently reported that the dialysis technique makes it possible to simultaneously monitor interstitial levels of NE and extraneuronal metabolites in the rabbit skeletal muscle [13]. Therefore, we consider it possible to elucidate extraneuronal NE metabolism and the role of COMT in its metabolism in the ischemic region. In the present study, we applied the dialysis technique to the heart of anesthetized rabbits and investigated myocardial interstitial levels of NE and its extraneuronal metabolites during coronary occlusion and reperfusion and examined the effect of COMT blockade on myocardial interstitial levels of NE and its metabolites.

## 2. Methods

### 2.1. Animal preparation

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Japanese white rabbits (2.5–3.2 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg iv). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). The rabbits were intubated and ventilated with room air mixed with oxygen. Body temperature was maintained at around 38 °C with a heating pad and lamp. Heart rate, arterial pressure, and electrocardiogram were monitored and recorded continuously. Heparin sodium (200 IU/kg) was first administered intravenously and then maintained with a continuous infusion (5–10 IU/kg/h) to prevent blood coagulation. With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to expose the heart. A small incision was made in the pericardium, and the dialysis probe was implanted in the region perfused by the left circumflex coronary artery (LCX) of the left ventricular wall. A snare was placed around the main branch of LCX to act as the occluder for later coronary occlusion. To ensure that the sampling area was in the ischemic region, we examined the color and motion of the ventricular wall during a brief occlusion and confirmed that the dialysis probe was correctly located. To avoid a preconditioning effect, the duration of occlusion was limited to within seconds.

### 2.2. Dialysis technique

Materials suitable for cardiac dialysis probes have been described in detail elsewhere [14]. Briefly, we designed a handmade long transverse dialysis probe. One end of a polyethylene tube (25-cm length, 0.5 mm OD, and 0.2 mm ID) was dilated with a 27-gauge needle (0.4 mm OD). Each end of the dialysis fiber (8-mm length, 0.31 mm OD, and 0.20 mm ID; PAN-1200 50 000 molecular weight cutoff, Asahi Chemical Japan) was inserted into the polyethylene tube and glued. We used a fine guiding needle (30-mm length, 0.51 mm OD, and 0.25 mm ID) for implantation of the dialysis probes. We connected a guiding needle to a dialysis probe with a stainless rod (5-mm length and 0.25 mm OD). At perfusion speed of 2 μl/min, in vitro recovery rates of NE, DHPG, normetanephrine (NMN), and 3-methoxy-4-hydroxyphenylglycol (MHPG) were 46 ± 8%, 48 ± 1%, 33 ± 3%, and 46 ± 2%, respectively (number of dialysis probes = 3) [15].

Dialysis probes were perfused with Ringer's solution at a speed of 2 μl/min using a microinjection pump (Carnegie Medicine CMA/100). Ringer's solution consisted of (in mM) 147.0 NaCl, 4.0 KCl, 2.25 $CaCl_2$. Sampling periods were 30 min (1 sampling volume = 60 μl) in control and 15 min (1 sampling volume = 30 μl) during occlusion and reperfusion. Each sample was collected in a microtube containing 3 μl of 0.1 N HCl to prevent amine oxidation. Based on the results of our previous study [1,2], we commenced the protocol followed by a stabilization period of 2 h. Taking into consideration the dead space between the dialysis fiber and sample tube, we sampled the dialysate.

Dialysate NE, DHPG, NMN, and MHPG concentrations were measured as indices of myocardial interstitial NE, DHPG, NMN, and MHPG levels. Furthermore, dialysate NE and DHPG were used as indices of COMT substrate, and dialysate NMN and MHPG as indices of COMT production. We used three distinct systems of high-performance liquid chromatography (HPLC) with electrochemical detection for the highly sensitive measurements: one for NE, one for DHPG, and one for NMN and MHPG measurement [16–18]. The mobile phase consisted of 1-octane-sulfonic acid sodium salt in phosphate buffer and methanol. In each HPLC system, the concentration of each component and the reference voltage were adjusted to the optimum condition. One-

third each of the dialysate sample was used for the measurement of NE, DHPG, and NMN and MHPG. Dialysate NE concentration was measured by the first HPLC after removing interfering compounds by the alumina procedure [14,16]. Dialysate DHPG concentration was measured by direct injection into the second HPLC [17]. Dialysate NMN and MHPG concentrations were measured by direct injection into the third HPLC [18]. The detection limits of NE, DHPG, NMN, and MHPG were 0.2, 0.2, 1, and 0.9 pg/injection.

### 2.3. Experimental protocols

After control sampling, we occluded the main branch of LCX for 60 min and then released the occluder. We continuously sampled dialysate from the ischemic region during 60 min of coronary occlusion and 15 min of reperfusion.

#### 2.3.1. Vehicle group (n=8)

We administered saline intraperitoneally as vehicle 120 min before control sampling. After control sampling, we observed the time course of dialysate NE, DHPG, NMN, and MHPG levels from the ischemic region during 60 min of coronary occlusion and 15 min of reperfusion.

#### 2.3.2. Entacapone group (n=8)

To elucidate role of COMT in the ischemia-induced changes in myocardial interstitial NE and its metabolites, we observed the effect of COMT inhibitor on dialysate NE, DHPG, NMN, and MHPG levels in the ischemic region. We administered intraperitoneally a COMT inhibitor entacapone (10 mg/kg; Orion Parma, Espoo, Finland) 120 min before control sampling. Entacapone was dissolved in phosphate-buffered saline, the pH of the solution was adjusted to 7.4. The route and dose of entacapone were selected to cause the full inhibition of soluble COMT in tissue [19]. After control sampling, we observed the time course of dialysate NE, DHPG, NMN, and MHPG levels with a similar protocol to that used in the vehicle group.

At the end of each experiment, the rabbits were killed with an overdose of pentobarbital sodium, and the implant regions were checked to confirm that the dialysis probes had been implanted within the cardiac muscle.

### 2.4. Statistical analysis

Hemodynamic and dialysate NE, DHPG, MHPG, and NMN responses to coronary occlusion in the presence and absence of COMT inhibitor were statistically analyzed by two-way analysis of variance with repeated measures on one factor [20]. When a statistically significant effect of coronary occlusion was detected as a whole, the Newman–Keuls test was applied to determine which mean values differed significantly from each other. When statistically significant effect of the COMT inhibitor was detected, the Newman–Keuls test was applied to determine which periods differed significantly between the vehicle and entacapone groups. Statistical significance was defined as $P < 0.05$. Values are presented as means ± SE.

## 3. Results

### 3.1. Time course of heart rate and arterial pressure

The time course of heart rate and mean arterial pressure is shown in Table 1. In the vehicle group, heart rate decreased after 15 min of occlusion, whereas in the entacapone group, heart rate increased after 30 min of occlusion. There was, however, no significant difference in heart rate between groups.

Coronary occlusion significantly decreased mean arterial pressure in both groups. In the entacapone group, mean arterial pressure was higher than those in the vehicle group at each sampling point, while changes in mean arterial pressure were similar to those in the vehicle group.

### 3.2. Dialysate NE levels in the ischemic region

Coronary occlusion significantly altered dialysate NE levels (Fig. 2). In the vehicle group, dialysate NE levels were 0.39 ± 0.07 nM in the control and increased after coronary occlusion. During 60 min of coronary occlusion, dialysate NE levels markedly increased and reached 165 ± 48 nM at 45–60 min of occlusion. After reperfusion, dialysate NE levels decreased to 62 ± 40 nM, although their

Table 1
Time course of heart rate and mean arterial pressure during coronary occlusion and reperfusion

| | Control | Coronary occlusion (min) | | | | Reperfusion (min) |
|---|---|---|---|---|---|---|
| | | 15 | 30 | 45 | 60 | 15 |
| *Heart rate (bpm)* | | | | | | |
| Vehicle group (n=8) | 252 ± 6 | 236 ± 7* | 238 ± 7* | 239 ± 6* | 237 ± 6* | 238 ± 6* |
| Entacapone group (n=8) | 246 ± 8 | 246 ± 8 | 251 ± 9* | 253 ± 7* | 253 ± 8* | 251 ± 9 |
| *Mean arterial pressure (mm Hg)* | | | | | | |
| Vehicle group (n=8) | 83 ± 3 | 71 ± 4* | 75 ± 3* | 76 ± 3* | 78 ± 2* | 76 ± 3* |
| Entacapone group (n=8) | 99 ± 4† | 85 ± 4*,† | 88 ± 5*,† | 88 ± 4*,† | 88 ± 4*,† | 86 ± 4*,† |

Values are means ± SE.
* $P < 0.05$ vs. control value.
† $P < 0.05$ vs. concurrent value of vehicle group.

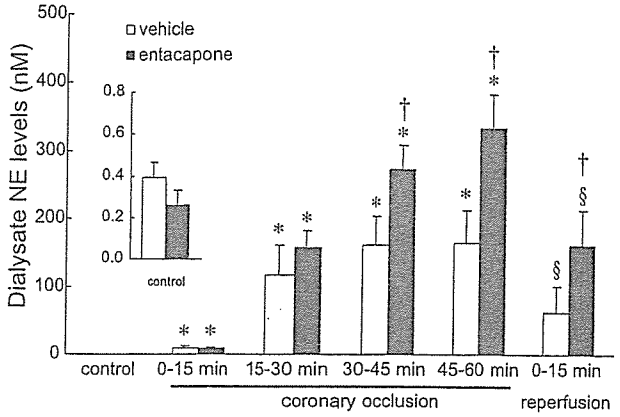


Fig. 2. Dialysate norepinephrine (NE) levels in the ischemic region. Values are means ± SE. *$P<0.05$ vs. control value, §$P<0.05$ vs. value at 45–60 min of occlusion, †$P<0.05$ vs. concurrent value of vehicle group.

levels were higher than those in the control. In the presence of entacapone, dialysate NE levels also markedly increased and reached 333 ± 51 nM at 45–60 min of occlusion. These increases in dialysate NE levels after 30 min of coronary occlusion were significantly enhanced by entacapone whereas entacapone did not change dialysate NE levels in the control (0.26 ± 0.07 nM). After reperfusion, dialysate NE levels decreased but remained higher than those in the vehicle group.

### 3.3. Dialysate DHPG levels in the ischemic region

Coronary occlusion significantly altered dialysate DHPG levels (Fig. 3). In the vehicle group, dialysate DHPG levels were 6.5 ± 0.5 nM in the control and did not change within 30 min of coronary occlusion. After 30 min of occlusion, dialysate DHPG levels gradually increased and reached 11.3 ± 1.1 nM at 45–60 min of occlusion. After reperfusion, dialysate DHPG levels further increased to 29.5 ± 2.6 nM. In the presence of entacapone, dialysate DHPG levels gradually increased during the ischemia and reached 22.9 ± 2.4 nM at 45–60 min of occlusion. After reperfusion, dialysate DHPG levels further increased to 52.6 ± 8.4 nM. In the presence of entacapone, dialysate DHPG levels in the control (9.9 ± 0.8 nM) and after reperfusion were higher than those in the vehicle group.

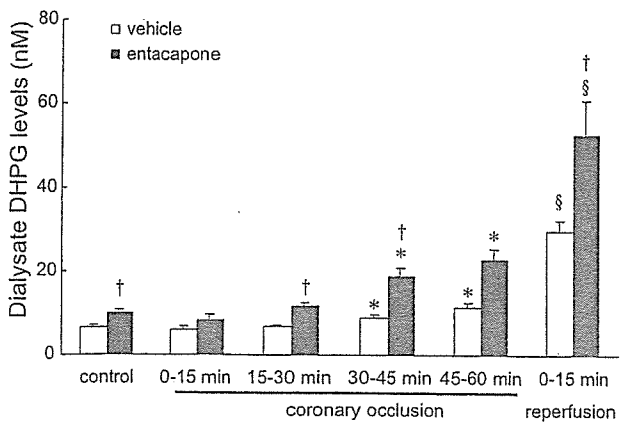


Fig. 3. Dialysate dihydroxyphenylglycol (DHPG) levels in the ischemic region. Values are means ± SE. *$P<0.05$ vs. control value, §$P<0.05$ vs. value at 45–60 min of occlusion, †$P<0.05$ vs. concurrent value of vehicle group.

### 3.4. Dialysate NMN levels in the ischemic region

Coronary occlusion significantly altered dialysate NMN levels (Fig. 4). In the vehicle group, dialysate NMN levels were 2.9 ± 0.4 nM in the control and increased after 30 min of occlusion and reached 9.3 ± 1.3 nM at 45–60 min of occlusion. After reperfusion, dialysate NMN levels further increased (11.9 ± 2.0 nM). Entacapone decreased dialysate NMN levels in the control to undetectable levels. Then dialysate NMN levels increased after 30 min of occlusion and reached 2.0 ± 0.4 nM at 45–60 min of occlusion. After reperfusion, dialysate NMN levels further increased to 4.1 ± 0.8 nM. Their NMN levels were lower than those in the vehicle group at each sampling point before, during, and after coronary occlusion.

### 3.5. Dialysate MHPG levels in the ischemic region

Coronary occlusion significantly altered dialysate MHPG levels (Fig. 5). In the vehicle group, dialysate MHPG levels were 3.9 ± 0.3 nM in the control. Dialysate MHPG levels transiently decreased 15–30 min after occlusion (3.6 ± 0.4 nM), but recovered after 30 min of occlusion. After reperfusion, dialysate MHPG levels increased to 5.5 ± 0.3 nM. Entacapone substantially decreased dialysate MHPG levels in the control (1.5 ± 0.4 nM). In the presence of entacapone, dialysate MHPG levels further decreased during occlusion and reached 0.6 ± 0.2 nM at 30–45 min of occlusion. After reperfusion, dialysate MHPG levels increased to 2.3 ± 0.5 nM. Their MHPG levels were lower than those in the

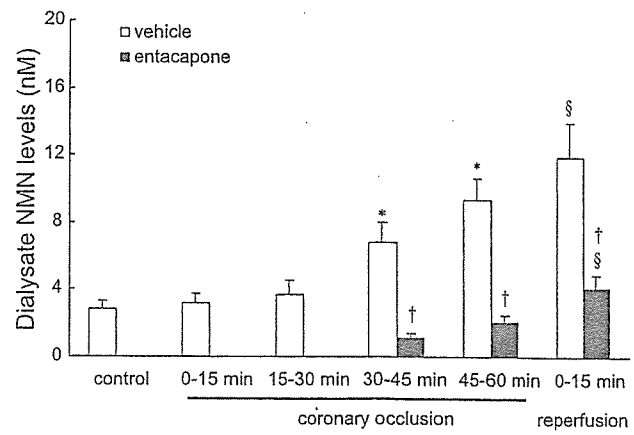


Fig. 4. Dialysate normetanephrine (NMN) levels in the ischemic region. Values are means ± SE. *$P<0.05$ vs. control value, §$P<0.05$ vs. value at 45–60 min of occlusion, †$P<0.05$ vs. concurrent value of vehicle group.

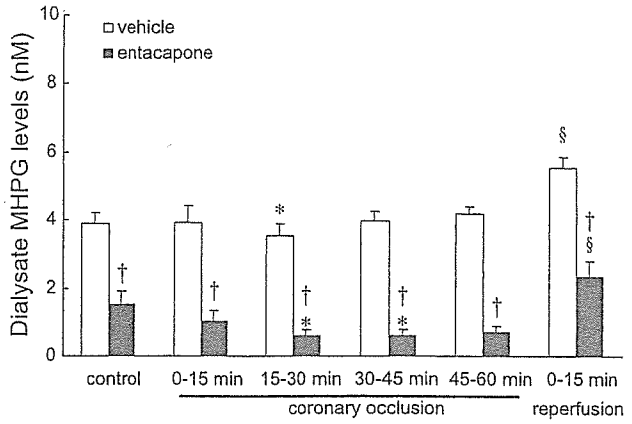


Fig. 5. Dialysate 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the ischemic region. Values are means ± SE. *$P<0.05$ vs. control value, §$P<0.05$ vs. value at 45–60 min of occlusion, †$P<0.05$ vs. concurrent value of vehicle group.

vehicle group at each sampling point before, during, and after coronary occlusion.

## 4. Discussion

Using the dialysis technique in the in vivo rabbit heart, we observed myocardial interstitial levels of NE and its neuronal and extraneuronal metabolites in the ischemic region and examined the contribution of extraneuronal NE degradation by COMT to myocardial interstitial NE levels. Our data demonstrate that COMT plays an important role in NE metabolism during 60 min of coronary occlusion and reperfusion.

### *4.1. Myocardial interstitial NE and its metabolites under control conditions*

The administration of entacapone did not alter myocardial interstitial NE levels in the control. Degradation of NE by COMT may play a minor role in the changes in myocardial NE levels [21]. In general, NE that is taken up by cardiac sympathetic nerve endings is repackaged or metabolized to DHPG by MAO. On the other hand, NE that is taken up via extraneuronal NE transport systems by extraneuronal cells is metabolized to NMN or MHPG by COMT [7–10,22,23] (Fig. 1). In the present study, entacapone increased DHPG in the myocardial interstitium but did not alter myocardial interstitial NE levels in the control. Myocardial interstitial levels of DHPG were about 16-fold higher than those of NE and about 2- to 3-fold higher than those of NMN in the control. Therefore, under physiological conditions, released NE could be largely taken up by cardiac sympathetic nerve endings via the $uptake_1$ carrier and transferred into stored vesicle or metabolized to DHPG by MAO. A smaller percentage of released NE, which escapes the synapses, is taken up by extraneuronal cells via the $uptake_2$ carrier and is metabolized to NMN by COMT. Compared with MAO, COMT could play a minor role on the degradation of released NE in the control.

After entacapone administration, increases in myocardial interstitial DHPG accompanied decreases in myocardial interstitial MHPG levels in the control. These metabolites of NE penetrate the cell membrane by diffusion [24]. Their values serve as indices of the neuronal and extraneuronal NE metabolism. COMT blockade suppressed the degradation of DHPG to MHPG. Therefore, the decrease in MHPG levels and increase in interstitial DHPG levels could be ascribed to inhibition of COMT by entacapone. In rabbit heart, we confirmed the existence of COMT activity with the main substrate of COMT being DHPG rather than NE.

### *4.2. Myocardial interstitial NE and its metabolites during coronary occlusion*

Myocardial interstitial NE levels markedly increased after 15 min of occlusion. In this phase, outward transport of NE via the $uptake_1$ carrier takes place from cardiac sympathetic nerve endings [2,3]. The marked increase in myocardial interstitial NE could be due to this non-exocytotic NE release and inhibition of neuronal reuptake via the $uptake_1$ carrier [2–4].

Entacapone augmented increases in myocardial interstitial NE levels after 30 min of coronary occlusion. This result indicates that COMT contributes to the degradation of myocardial interstitial NE in this phase. Neuronal reuptake via the normal mode of $uptake_1$ is dependent on the sodium gradient between the intra- and extraneuronal space [25]. Neuronal degradation of released NE via neuronal uptake cannot be expected in this phase because of a reduced sodium gradient [3,25]. Moreover, NE spillover into the bloodstream is decreased due to reduced myocardial blood flow [1]. On the other hand, extraneuronal NE uptake operates independently of the sodium gradient [26]. Burgdorf et al. [27] demonstrated that the extraneuronal monoamine transporter is activated during metabolic distress such as low flow ischemia. Previous investigations with similar preparations suggested that ketamine augments ischemia-induced NE accumulation by inhibition of extraneuronal uptake [28,29]. We consider that substantial NE in the myocardial interstitium is taken up alternatively by extraneuronal cells via the $uptake_2$ carrier and is metabolized to NMN by COMT in this phase. Thus, COMT activity plays an important physiological role in NE degradation in the ischemic period.

Myocardial interstitial NMN levels increased after 30 min of occlusion. Entacapone decreased basal myocardial interstitial NMN levels and suppressed the ischemia-induced increase in myocardial interstitial NMN levels. This suppression is consistent with the finding that COMT contributes to the degradation of myocardial interstitial NE via extraneuronal NE uptake. Furthermore, even in the presence of entacapone, myocardial interstitial NMN levels increased after 30 min of occlusion. An increase in interstitial NE levels may overcome the inhibition of COMT by

entacapone. Although COMT could play a minor role in the degradation of released NE in the control, a substantial increase in myocardial NE may cause the high affinity of the extraneuronal COMT system. In the control period, an extraneuronal COMT system may contribute to DHPG degradation, whereas in the ischemic period, both neuronal NE uptake and MAO activities may be suppressed by ischemia and alternatively the extraneuronal COMT system may promote NE degradation based on the fact that the myocardial interstitial NE levels in the ischemic period were 10-fold higher than those of DHPG. Therefore, we consider that increases in myocardial interstitial NE levels shift the main substrate of COMT from DHPG to NE.

The relationship between DHPG and MHPG supports our interpretation. In the control, entacapone increased myocardial interstitial DHPG levels and decreased myocardial interstitial MHPG levels. In contrast, in the ischemic period, increases in myocardial interstitial DHPG levels were not associated with increases in myocardial MHPG levels, but increases in myocardial NE levels accompanied increases in myocardial NMN. Thus, both DHPG and NE are metabolized by the extraneuronal COMT system, but the amount of NMN and MHPG may be dependent on the concentration of their substrate. Thus, there was a clear difference in the main metabolite by COMT between the non-ischemic and ischemic periods. The main metabolite by COMT was NMN rather than MHPG in the latter [30]. These results are limited to ischemia within 60 min because prolonged ischemia accompanies the structural membrane defects, and other mechanisms for NE release and degradation may be involved [31].

### *4.3. Myocardial interstitial NE and its metabolites after reperfusion*

Myocardial interstitial NE levels decreased after reperfusion although they were higher than those in control. On the other hand, myocardial interstitial DHPG levels increased after reperfusion. The uptake$_1$ carrier resumes normal transport function after reperfusion [2,4]. The inward NE transport via the uptake$_1$ carrier could contribute to the decrease in myocardial interstitial NE levels. The increase in axoplasmic NE by uptake and the recovery of MAO activity could increase myocardial interstitial DHPG levels [2]. Thus, neuronal degradation by MAO contributes to decreasing myocardial interstitial NE after reperfusion.

Reperfusion caused a decrease in myocardial interstitial NE levels and an increase in myocardial NMN levels, both of which changes were suppressed by administration of entacapone. During the early reperfusion period, COMT activity promotes the degradation of NE. Myocardial interstitial NE levels were higher than those in the control. Therefore, during the ischemic and reperfusion periods, these higher NE levels in myocardial interstitium may serve as an effective substrate of COMT for NE degradation via extraneuronal NE uptake.

During the reperfusion period, increases in myocardial interstitial DHPG accompanied increases in myocardial interstitial MHPG levels. Furthermore, administration of entacapone suppressed both of these changes. Myocardial interstitial DHPG levels were similar to myocardial interstitial NE levels. These data suggest that COMT activity promotes the degradation of DHPG. Alternatively, higher NE level in myocardial interstitium may produce MHPG via COMT activity. Recently, we demonstrated in rabbit skeletal muscle that local administration of higher NE increased dialysate NMN but not MHPG levels, whereas local administration of higher DHPG increased dialysate MHPG levels [19]. Therefore, higher NE and DHPG levels serve as the substrate of COMT and independently yield NMN and MHPG during the reperfusion period. Thus, COMT activity plays an important physiological role in the reperfusion period.

### *4.4. Methodological considerations*

In the presence of a high concentration of entacapone, mean arterial pressure was higher than that in the vehicle group at each sampling point before, during, and after coronary occlusion, but changes in mean arterial pressure were similar to those in the vehicle group. In the previous and present studies, intraperitoneal administration of entacapone did not alter control dialysate NE levels from skeletal muscle and myocardium [19]. In humans, entacapone did not alter plasma catecholamine levels or hemodynamics at rest or during exercise [32]. The influence of entacapone on pressure-regulating peptides remains unclear. An increase in mean arterial blood pressure might decrease dialysate NE levels through a baroreflex mechanism. Furthermore, baroreflex-independent and non-exocytotic NE efflux leads to high NE levels in the myocardial interstitium of ischemic regions, making it unlikely that hemodynamic change contributes to the removal of accumulated NE during myocardial ischemia.

Two major classes of COMT have been defined on the basis of their location: a soluble, cytosolic form and a membrane-bound form [33]. Entacapone inhibits both classes of COMT. The soluble, cytosolic form is generally assumed to be the predominant form of the enzyme. The membrane-bound form has been suggested to be responsible for O-methylation at low and physiologically relevant concentrations of the catecholamine neurotransmitters, whereas the soluble, cytosolic form predominates under conditions that lead to saturation of the membrane-bound form [33]. In the present study, myocardial interstitial norepinephrine levels reached 100–1000 times the normal plasma concentrations after 15 min of occlusion. Thus, a soluble, cytosolic form could contribute to the observed decrease in myocardial interstitial NE levels in the ischemic region.

## 5. Conclusion

Under physiological condition, extraneuronal enzymatic degradation by COMT plays a minor role on the inactivation

of myocardial interstitial NE. Under ischemic conditions, however, myocardial interstitial NE levels are markedly increased by ischemia. Normal transport by the uptake$_1$ carrier is impaired and NE spillover into the bloodstream is decreased due to the reduction of myocardial blood flow, but extraneuronal enzymatic degradation by COMT contributes to the decrease in myocardial interstitial NE levels in the ischemic region.

## Acknowledgements

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# Local Delivery of Argatroban for the Prevention of Restenosis After Coronary Balloon Angioplasty

## — A Prospective Randomized Pilot Study —

Tomonori Itoh, MD; Hiroshi Nonogi, MD; Shunichi Miyazaki, MD; Akira Itoh, MD; Satoshi Daikoku, MD; Isao Morii, MD; Yoichi Goto, MD for the 3D-CAT investigators

**Background** Effective pharmacological prevention of restenosis using the systemic administration of various drugs that were effective for the prevention of restenosis in experimental studies has not been reported. The purpose of this study was to evaluate whether the local delivery of a potent thrombin inhibitor, argatroban, using a local drug delivery device would prevent restenosis after plain old balloon angioplasty (POBA).

**Methods and Results** Seventy patients with chronic coronary artery disease requiring POBA were randomly assigned to wither the control group (n=35) or the argatroban group (n=35). In the argatroban group, argatroban was administered intravenously for 30 min before the POBA and intracoronarily into the dilated site using a Dispatch™ catheter immediately after the POBA, followed by a postoperative intravenous infusion for 4 h. The angiographical lesion restenosis and clinical restenosis rates at follow-up were significantly lower in the argatroban group (27% and 14%) than in the control group (56% and 37%; p=0.02 and p=0.03, respectively). There was no major complication during the procedure.

**Conclusion** The local delivery of argatroban is safe and effective in preventing restenosis after balloon angioplasty. (*Circ J* 2004; **68:** 615–622)

**Key Words:** Coronary angioplasty; Direct thrombin inhibitor; Local drug delivery; Restenosis

The clinical efficacy of coronary balloon angioplasty (plain old balloon angioplasty: POBA) is limited by restenosis, which occurs in 30–50% of cases despite a successful procedure[1–4] However, in previous clinical trials[5–9] there has not been effective pharmacological prevention of restenosis using the systemic administration of various drugs that were found to be effective for the prevention of restenosis in experimental studies. One of the major factors in the failure of restenosis prevention in these clinical trials could be that the systemic administration of drugs resulted in a concentration at the site of a balloon injury that was too low. Accordingly, it has been anticipated that the local delivery of a drug at a high concentration may reduce the restenosis rate after POBA. However, the pharmacological prevention of restenosis using a local drug delivery system has not yet been tested in clinical trials except for one small-scale trial[10]

It was recently reported that the messenger RNA (mRNA) of a thrombin receptor is expressed in medial smooth muscle cells in the very early phase after a balloon catheter injury (within 6 h)[11,12] Moreover, pre-treatment with hirudin (a direct thrombin inhibitor) was found to reduce vascular lesion development after balloon injury in experimental studies[13,14] Thus, it is thought that restenosis may be prevented or minimized by the local administration of a direct thrombin inhibitor. Argatroban is a direct thrombin inhibitor that has a more potent inhibitory effect on fibrin- or clot-incorporated thrombin than other thrombin inhibitors such as heparin and hirudin[15,16] Tomaru et al reported that the local delivery of argatroban using a double-balloon catheter reduced intimal thickening after balloon injury in an experimental study[17] Accordingly, we conducted a prospective, randomized, controlled clinical trial to assess the effect of the local delivery of argatroban as a direct thrombin inhibitor using a Dispatch™ catheter system[18] (SIMED Life Systems, Inc, Maple Grove, MN, USA) in the prevention of restenosis after percutaneous coronary intervention (PCI).

## Methods

*Study Protocol*

Between March 1995 and May 1997, 70 patients who required coronary revascularization were registered in the present trial (Drug Delivery Device in Coronary Balloon Angioplasty Trial: 3D-CAT) at the National Cardiovascular Center. The 3D-CAT is a randomized controlled pilot trial for prevention of restenosis after coronary balloon angioplasty conducted at a single center. The inclusion criterion was that the patient was scheduled for elective POBA with a balloon size equal to or larger than 2.75 mm. All of the patients had ischemic chest pain or evidence of ischemia diagnosed by a thallium-201 or treadmill exercise test. The patients were randomly assigned to 2 groups

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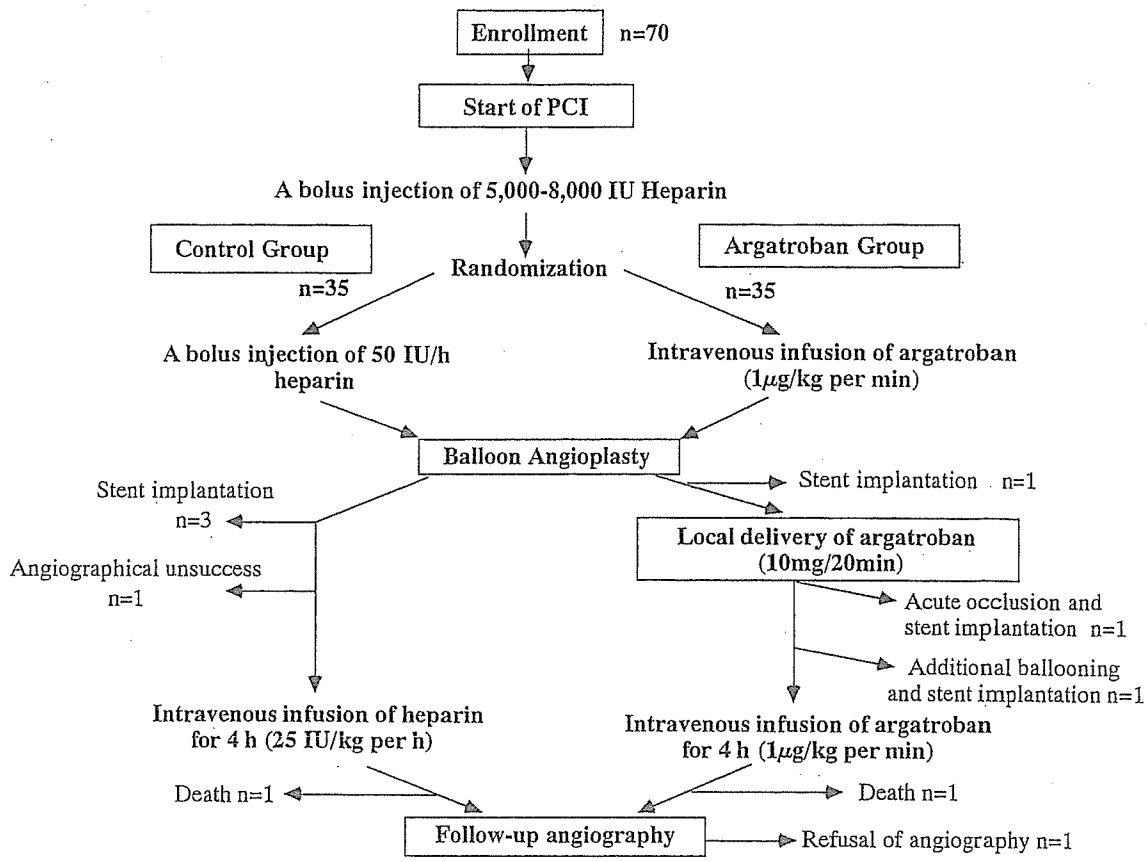


Fig 1. Study protocol and patient flow chart. A bolus of 5,000–8,000 IU heparin was injected intravenously at the start of the percutaneous coronary intervention (PCI) procedure. Patients were randomly assigned to 2 groups: the control group and the argatroban group receiving local delivery of argatroban via a Dispatch™ catheter after PCI. Control group: PCI was performed; the patients received a bolus injection of heparin during the procedure and an intravenous infusion of heparin for 4 h after angioplasty. Argatroban group: intravenous infusion of argatroban was started 30 min before the PCI, followed by local delivery of argatroban into the dilated site using a Dispatch™ catheter, and the postoperative treatment of intravenous infusion of argatroban for 4 h.

according to consecutive sealed envelopes; the control group (n=35) underwent a conventional method of POBA, and the argatroban group (n=35) had the addition of local delivery of argatroban. The exclusion criteria were (1) more than 80 years old or less than 20 years old, (2) a target lesion in a non-protected left main coronary artery, (3) a total occlusive lesion equal to TIMI 0-1 flow, (4) a severely calcified lesion, (5) a diffuse lesion, (6) a target vessel with severe proximal tortuosity, (7) a lesion that restenosed more than once, (8) a bypass graft vessel, (9) an indication for a new device (eg, directional coronary atherectomy, stent, rotational atherectomy or laser ablation), (10) poor left ventricular function (ejection fraction <40%), (11) patients receiving warfarin, (12) patients receiving an intravenous infusion of heparin, (13) a history of gastrointestinal bleeding, thrombocytopenia, or coagulopathy, (14) a history of stroke within the preceding 3 months, (15) acute myocardial infarction within the previous month, (16) patients undergoing thrombolysis within the past 24 h, (17) pregnancy, and (18) other major illness including renal failure and liver dysfunction. Informed consent was obtained from each patient.

*PCI Procedure and Adjunctive Therapy*

Coronary angiography was performed using the Judkins method, and a bolus of 5,000–8,000 IU heparin was given intravenously after vascular access had been established. In the control group, the POBA was performed in a standard way with a bolus injection of heparin (50 U/kg per h) during the procedure, followed by an infusion of heparin (25 U/kg per h) for 4 h after the POBA. In the argatroban group, an intravenous infusion of argatroban was given (1 μg/kg per min) 30 min before POBA, followed by the local delivery of argatroban (10 mg/20 min) into the dilated site using the Dispatch™ catheter (SIMED Life Systems) after the successful POBA. Postoperatively, the patients received an intravenous infusion of argatroban (1 μg/kg per min) for 4 h (Fig 1). All patients received both Ca antagonist and 81–162 mg of aspirin before the POBA until the follow-up angiography. In addition, β-blockers, long-acting isosorbide dinitrates or nicorandil was administered at the discretion of the treating physician before the POBA until the follow-up coronary angiography. Clinical success of the POBA was defined as angiographic success (residual stenosis <50%) without a major complication (death, myocardial infarction, or emergency coronary-artery bypass surgery) during hospitalization.

*Quantitative Coronary Angiographic Analysis*

All angiograms were analyzed by a computer-assisted system of quantitative coronary angiographic analysis (QCA; Cardiovascular Measurement System Ver. 3.0

Table 1 Baseline Clinical Characteristics of the Study Patients

| | Control group (n=35) | Argatroban group (n=35) | p value |
|---|---|---|---|
| Age (years) | 61±8 | 61±8 | NS |
| M/F | 29/6 | 27/8 | NS |
| Risk factors | | | |
| BMI | 23.6±2.0 | 23.5±2.6 | NS |
| Diabetes mellitus | 18 | 12 | NS |
| Hypertension | 19 | 23 | NS |
| Total cholesterol (mg/dl) | 185±32 | 198±36 | NS |
| Prior MI | 13 | 8 | NS |
| Ejection fraction (%) | 57±10 | 58±13 | NS |
| Diseased coronary vessels | | | |
| 1-vessel disease | 23 | 23 | NS |
| 2-vessel disease | 11 | 12 | |
| 3-vessel disease | 1 | 0 | |
| Target vessel | | | |
| LAD/LCX/RCA | 18/13/4 | 16/16/3 | NS |

BMI, body mass index; MI, myocardial infarction; LAD, left anterior desending artery; LCX, left circumflex artery; RCA, right coronary artery.

Table 2 Baseline Lesion Characteristics of the Study Patients

| | Control group (n=35) | Argatroban group (n=35) | p value |
|---|---|---|---|
| ACC/AHA classification | | | |
| A | 5 | 5 | |
| B | 29 | 29 | NS |
| C | 1 | 1 | |
| De novo lesion | 32 (94%) | 28 (80%) | NS |
| Reference vessel diameter (mm) | 2.89±0.46 | 2.89±0.39 | NS |
| Minimal lumen diameter (mm) | 0.86±0.24 | 0.83±0.19 | NS |
| Lesion length (mm) | 6.00±3.69 | 5.25±3.77 | NS |
| Lesion characteristics | | | |
| Eccentricity | 29 (83%) | 27 (77%) | NS |
| Calcification | 12 (34%) | 16 (46%) | NS |
| Ostial lesion | 4 (11%) | 5 (14%) | NS |
| Proximal tortuosty | 2 (6%) | 1 (3%) | NS |
| Angled lesion | 4 (11%) | 4 (11%) | NS |
| Bifurcation | 7 (20%) | 6 (17%) | NS |

(CMS), Medical Imaging Systems Inc, Leiden, the Netherlands). CAG was performed before, immediately after, and 3 months after the POBA (follow-up) as described in detail elsewhere[19] All angiographic analyses were performed in a blinded fashion by an experienced physician. The % diameter stenosis (%DS) and minimal lumen diameter (MLD) of the target lesion were determined quantitatively. The diameter of a Judkins catheter was measured using a precision micrometer (No. 293-421-20; precision 0.001 mm, Mitutoyo Co, Kawasaki, Japan) to obtain a calibration factor in the 'Free French' mode in the image calibration of the CMS program. The calibration factor (CF) was adjusted between 0.08 and 0.1 mm/pixel using digital zoom according to the CMS manual[20] The complex edit mode (gradient field transform: GFT) was used in the case of a complex lesion, as described in detail elsewhere[21]

Angiographic restenosis after POBA was defined as a %DS greater than 50% on the follow-up angiogram. Clinical restenosis was defined as the recurrence of ischemia and/or target lesion revascularization within the period before the follow-up angiography.

*Endpoints*

The following endpoints were prospectively defined. Restenosis was the primary endpoint. Secondary endpoints included death, acute myocardial infarction (symptoms, ECG changes, and creatine kinase >twice the upper normal limit) and coronary revascularization (coronary bypass surgery, or repeated POBA and/or coronary stenting). Repeat revascularization of the target lesion (target lesion revascularization) was defined as angioplasty or bypass surgery performed because of restenosis of the target lesion in association with recurrent angina, objective evidence of myocardial ischemia, or both. The principal safety endpoints were abrupt vessel closure, stroke, major bleeding, or the need for vascular surgery. Major bleeding was defined as intracranial hemorrhage or overt bleeding associated with a decrease in hemoglobin of more than 5 g/dl.

*Statistical Analyses*

The data are presented as mean±SD (standard deviation). Differences in angiographical parameters (%DS and MLD) between the 2 groups before POBA, immediately after all procedure and during the follow-up were compared by unpaired t-test. Statistical comparisons of differences in categorical data between the 2 groups were performed using the chi-square test. Differences were considered significant when $p<0.05$. The clinical follow-up analyses were performed on an intention-to-treat basis and on-treatment-analyses. Moreover, angiographic follow-up analyses were performed using on-treatment-analyses.

Table 3 In-Hospital Outcomes of the Study Patients

| | Control group (n=35) | Argatroban group (n=35) | p value |
|---|---|---|---|
| Stent required (%) | 3 (8.6) | 3 (8.6) | NS |
| Acute occlusion (%) | 0 (0) | 1 (2.8) | NS |
| Additional ballooning (%) | 0 (0) | 1 (2.8) | NS |
| Angiographical nonsuccess (%) | 1 (2.8) | 0 (0) | NS |
| Acute myocardial infarction (%) | 0 (0) | 0 (0) | NS |
| Emergency CABS (%) | 0 (0) | 0 (0) | NS |
| Death (%) | 0 (0) | 0 (0) | NS |

CABS, coronary artery bypass surgery; MI, myocardial infarction.

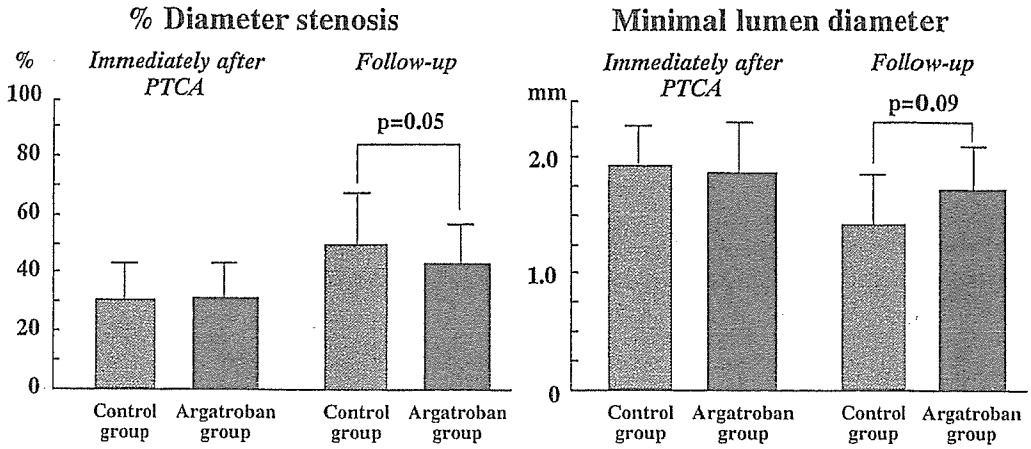


Fig 2. There were no significant differences between the 2 groups in % diameter stenosis or minimal lumen diameter immediately after PCI. Angiographic parameters including % diameter stenosis and minimal lumen diameter were marginally better in the argatroban group than in the control group at follow-up.

## Results

*Patient Population (Fig 1)*

Four patients in the argatroban group were excluded from the follow-up CAG; 1 underwent stent implantation because of a major coronary dissection before the local delivery of argatroban, 1 had an abrupt vessel closure during the local delivery of argatroban, 1 required additional ballooning and stent implantation, and 1 refused to undergo the follow-up CAG with negative exercise thallium-201 stress imaging. Four patients in the control group were also excluded from the follow-up CAG: 3 required stent implantation because of major coronary dissection after the balloon angioplasty, and 1 had residual %DS >50% (angiographically unsuccessful). During the course of the study, 2 patients died suddenly (control 1, argatroban 1) before the follow-up angiography; the 1 in the argatroban group had cardiac sudden death after balloon angioplasty on day 60 (the patient had an old myocardial infarction with left ventricular dysfunction) and the patient in the control group died suddenly on day 60 after the balloon angioplasty (suspected rupture of a thoracic aortic aneurysm). In total, 10 patients (5 in each group) were excluded from the follow-up angiography.

*Baseline Clinical and Lesion Characteristics*

Tables 1 and 2 summarize the baseline clinical and lesion characteristics; there were no significant differences between the 2 groups in this study.

*In-Hospital Outcome*

The in-hospital outcomes are summarized in Table 3. An acute occlusion in the treated segment during the local delivery of argatroban using a Dispatch™ catheter was observed in 1 patient, requiring implantation of a Palmaz-Schatz stent. There were no major complications during the procedure in either group.

*Quantitative CAG Analyses at Follow-up*

Fig 2 compares the results of the angiographic analyses between the 2 groups. There were no significant differences between the 2 groups in %DS (Control group: 30.9±10.9%, Argatroban group: 31.7±9.6%) or MLD (Control group: 1.95±0.3 mm, Argatroban group: 1.92±0.35 mm) immediately after procedure. However, after 3 months, the angiographic parameters of %DS (Control group: 51.3±16.2%, Argatroban group: 43.5±14.6%) and MLD (Control group: 1.36±0.46 mm, Argatroban group: 1.57±0.47 mm) were marginally better in the argatroban group than in the control group (p=0.05 and p=0.09, respectively). The mean difference in coronary MLD (net gain) between the post-procedure and follow-up angiograms was 0.51±0.44 mm in the control group, and 0.72±0.50 mm in the argatroban group (p=0.09).

*Restenosis Rates, Target Lesion Revascularization, and Clinical Follow-up Data*

Fig 3 compares the restenosis rates in the 2 groups. The lesion restenosis (%DS >50%) rates were 27% in the argatroban group and 56% in the control group (p=0.02). The clinical restenosis rates were 14% in the argatroban group and 37% in the control group (intention-to-treat analysis; Table 4, p=0.03). The target lesion revascularization rates were 14% in the argatroban group and 34% in the control

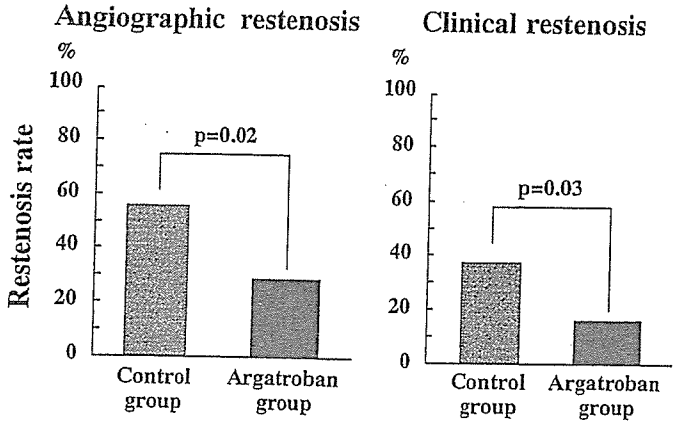


Fig 3. Angiographic restenosis occurred in 5 of the 30 patients in the argatroban group (27%) and 12 of the 30 patients in the control group (56%). Clinical restenosis occurred in 5 of the 35 patients in the argatroban group (14%) and 13 of the 35 in the control group (37%).

Table 4 Clinical Outcome at Follow-up of the Study Patients

| | *Control group (n=35)* | *Argatroban group (n=35)* | *p value* |
|---|---|---|---|
| *Clinical restenosis* | *13** | *5* | *0.03* |
| *Target vessel revascularization* | *12* | *5* | *0.05* |
| *Vasospastic angina* | *0* | *1* | *NS* |
| *Myocardial infarction* | *0* | *0* | *NS* |
| *Death* | *1* | *1* | *NS* |
| *Any clinical event* | *14* | *7* | *0.07* |

*Clinical restenosis included recurrence of ischemia and/or angina and target vessel revascularization.*
**Includes one case of recurrence of ischemia (silent) without target vessel revascularization.*

Table 5 Clinical Outcome at Follow-up of the Study Patients According to on-Treatment Analysis

| | *Control group (n=31)* | *Argatroban group (n=32)* | *p value* |
|---|---|---|---|
| *Clinical restenosis* | *12** | *5* | *0.03* |
| *Target vessel revascularization* | *11* | *4* | *0.06* |
| *Vasospastic angina* | *0* | *1* | *NS* |
| *Myocardial infarction* | *0* | *0* | *NS* |
| *Death* | *1* | *1* | *NS* |
| *Any clinical event* | *13* | *7* | *0.08* |

*Clinical restenosis included recurrence of ischemia and/or angina and target vessel revascularization.*
**Includes one case of recurrence of ischemia (silent) without target vessel revascularization.*

group (intention-to-treat analysis; Table 4, p=0.05). Moreover, Table 5 shows the clinical outcome at follow-up of the study patients on-treatment-analysis. Seven cases (6 stent implantations and 1 unsuccessful procedure during initial angioplasty) were excluded in Table 5 according to on-treatment-analysis. The clinical restenosis rates at follow-up were 17% (n=5) in the argatroban group and 40% (n=12) in the control group according to on-treatment-analysis after exclusion of 10 cases (n=30, respectively; p=0.04). The details of those 10 cases are as follows: 6 stent implantations during procedure, 1 unsuccessful procedure, 2 deaths, and 1 refusal of follow-up CAG.

## Discussion

*Previous and Present Trials Regarding the Prevention of Restenosis*

No definitively effective prevention of restenosis by systemic administration of drugs has been observed in previous clinical trials. Several types of drug therapy, such as anticoagulants (heparin, warfarin) and antiplatelet therapy (aspirin, dipyridamole, ticlopidine, prostacyclin, and thromboxane $A_2$ inhibitor), fish oil, and steroids have failed to reduce the restenosis rate in most clinical trials[22–24] Recently, trapidil and cholesterol-lowering agents have been shown to be promising in preventing restenosis after coronary angioplasty[25] but patients must take these drugs for several months after angioplasty.

In contrast, coronary stenting has been shown to be effective in preventing restenosis after coronary angioplasty[26,27] and the drug eluting stent has been developed in recent years[28] Nevertheless, adjunctive anticoagulation and/or antiplatelet therapy is required for 1 month after coronary stenting, resulting in occasional bleeding complications. Accordingly, a new procedure with a low rate of adverse effects and no need for adjunctive therapy after discharge has been sought. In the present randomized, controlled study, local delivery plus intravenous infusion of argatroban reduced both the angiographic and clinical restenosis rates after coronary angioplasty. There was no increase in bleeding risk with the argatroban treatment. The restenosis rate in the argatroban group in this trial (27%) was similar to that in the stent group of the STRESS trial (32%; NS)[27] despite the fact that the reference vessel diameter was smaller (2.89±0.39 mm) than that in the STRESS trial (3.03±0.42 mm; p=0.07). The restenosis rate in the