EFFECTS OF EXCESSIVE NORADRENALINE ON CARDIAC
MITOCHONDRIAL CALCIUM TRANSPORT AND
OXIDATIVE PHOSPHORYLATION

Takahito Sone, M.D., Yutaka Miyazaki, M.D., Kouichi Ogawa, M.D.
and Tatsuo Satake, M.D.

Mitochondrial oxidative phosphorylation, calcium transport activity and
calcium content were investigated in dog hearts injured by excessive noradren-
aline (NA). Diffuse cardiac injury was produced by a 5-hour infusion of NA
(2 or 5 μg/kg/min), and the injury was evaluated based on ECG and hemo-
dynamic changes. Mitochondrial calcium uptake and binding activities
measured in the presence of ATP showed no significant differences between
the control and NA groups. However, the calcium content of heart mito-
chondria isolated from the NA groups, state 3 respiration and the respiratory
control index were significantly depressed without any change in the ADP/O
ratio. These results suggest that excessive NA causes the intracellular calcium
overload and the depression of mitochondrial respiration, and the both of
these changes may play a key role in the pathogenesis of myocardial injury
through the insufficient control of cytosolic calcium levels.

Recently, it has been suggested that the
amount of noradrenaline stored in the
heart is large enough to induce cellular damage
and that high local concentrations of free nor-
adrenaline released from its stores may play an
important role in the development of several pathologic conditions including the myocardial
infarction process. In addition, the nor-
adrenaline concentrations locally available in the
heart under these pathologic conditions have
been reported by Reichenbach et al. to be com-
parable to the concentrations achieved by exoge-
nous administration in an experiment producing
myocardial lesions. From this point of view, it is

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Second Department of Internal Medicine, Faculty of Medicine, University of Nagoya, Tsuruma, Showa-ku,
Nagoya 466, Japan.
Mailing address: Takahito Sone, M.D., Second Department of Internal Medicine, Faculty of Medicine, Uni-
versity of Nagoya, Tsuruma, Showa-ku, Hagiyo 466, Japan

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The present study was undertaken to clarify the mechanism of noradrenaline-induced myocardial damage by evaluating the changes in the mitochondrial calcium transport activity and oxidative phosphorylation, together with the calcium and magnesium contents in mitochondria.

MATERIALS AND METHODS

Adult mongrel dogs of either sex weighing 8 to 12 kg were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), supplemented by small additional amounts sufficient to maintain anesthesia. The animals were given artificial respiration mechanically through a cuffed endotracheal tube, and arterial PO₂ and PCO₂ were maintained within the normal range by adjusting the inspired PO₂ and altering the respirator settings for rate and tidal volume. Arterial pH was maintained between 7.35 and 7.45 by the administration of sodium bicarbonate when necessary. A femoral vein was cannulated for infusions and femoral artery for blood pressure and gas analysis.

Twenty-eight dogs were divided into 3 groups. Dogs in the control group were infused with 500 ml of saline for 5 hours. Dogs in the other two groups, global myocardial injury was produced according to the procedure of Schenk et al. Noradrenaline (Sigma Chemical Co.) dissolved in the isotonic saline was infused at a rate of 2 μg/kg/min in the group 1 and 5 μg/kg/min in the group 2 for 5 hours. The total volume of the infusion was adjusted to 500 ml and infused using a constant infusion pump. The dogs were sacrificed at the end of the infusion, and the hearts were quickly excised and placed in an ice-cold mitochondrial isolation medium consisting of 0.25 M sucrose and 50 mM Tris-HCl (pH 7.4). Heart mitochondria were prepared by a method of Peng et al. using an EDTA free extraction medium to retain the endogenous calcium. The final mitochondrial pellet was suspended in an hemogenization solution consisting of 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4). The protein concentration was determined using the biuret method.

Mitochondrial oxidative phosphorylation was studied polarographically at 30°C using an oxygen electrode and a closed cell (YSI Model 53). The rate of oxygen consumption (state 3 and state 4), the respiratory control index (RCI) and the ratio of ADP phosphorylated to the amount of oxygen consumed (ADP/O) were measured immediately after the preparation of mitochondria. The assay mixture contained 0.3 M mannitol, 10 mM potassium phosphate, 10 mM KCl, 2.5 mM MgCl₂, 0.25 mM EDTA, pH 7.4, 10 mM succinate and approximately 2 mg mitochondrial protein in a total volume of 3 ml. Following the addition of the substrate and mitochondria, the active respiration (state 3) was initiated by the addition of ADP.

Mitochondrial calcium uptake and binding were determined using the millipore filter method described by Harigaya and Schwartzenberg. The medium used to measure binding contained the following: 20 mM Tris-maleate (pH 6.8), 100 mM KCl, 10 mM MgCl₂, 2 mM ATP, 0.05 mM hexadecyltrimethylammonium chloride and about 0.4 mg/ml mitochondrial protein at 25°C. To measure the calcium uptake, succinate (5 mM) and inorganic phosphate (5 mM) were added to facilitate the mitochondrial respiration. Mitochondrial protein concentration was reduced to 0.2 mg/ml, 45CaCl₂ increased to 0.1 mM and the reaction was carried out at 37°C. In both measurements, the reaction was initiated by the addition of mitochondrial protein and terminated at various times by millipore filtration (HA 0.45 μ). Ca²⁺ activity in the filtrate was measured using a liquid scintillation counter (Beckman type LS 7500).

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Figure 1. The percentage changes in mean arterial pressure during the 5-hour infusions. Hatched rectangles are the control group, and the striped and black rectangles are the group 1 and the group 2, respectively. Typical electrocardiographic changes (lead II) in noradrenaline infused groups are shown above the graph. In the control group, no significant change of QRS complex was observed throughout the experiment.
TABLE I OXIDATIVE PHOSPHORYLATION BY CARDIAC MITOCHONDRIA ISOLATED FROM EACH GROUP

<table>
<thead>
<tr>
<th>Group</th>
<th>State 3 (n atoms O/min/mg protein)</th>
<th>State 4 (n atoms O/min/mg protein)</th>
<th>RCI</th>
<th>ADP:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>270 ± 10</td>
<td>50 ± 2</td>
<td>5.2 ± 0.3</td>
<td>1.94 ± 0.04</td>
</tr>
<tr>
<td>Group 1 (n = 8)</td>
<td>230 ± 13</td>
<td>49 ± 2</td>
<td>4.6 ± 0.2</td>
<td>1.88 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Group 2 (n = 8)</td>
<td>190 ± 14</td>
<td>47 ± 3</td>
<td>4.3 ± 0.2</td>
<td>1.80 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td>n.s.</td>
<td>p &lt; 0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Five-hour treatment with noradrenaline (Group 1: 2 µg/kg/min; Group 2: 5 µg/kg/min) caused significant decreases in state 3 respiration and the respiratory control index (RCI) in a dose-dependent manner. While neither state 4 respiration nor the ratio of ADP phosphorylated to the amount of oxygen consumed (ADP:0) were affected by noradrenaline treatment. All values are represented as the mean ± SE of the number of samples in the parentheses. P value are calculated by using the non-paired t-test between the control and the Group 1 or 2.

The measurement of mitochondrial calcium and magnesium contents were performed by a modified method of Shen and Jennings. Mitochondrial samples (10–20 mg protein) were underwent wet ashing by 8 ml of conc HNO₃ and 2 ml of conc H₂SO₄ at 80°C for several hours. Addition of conc HNO₃ was supplemented as required until the reaction was complete. After cooling, 5 ml of glass-distilled water was added and the resulting solution was used for the assay of calcium and the magnesium contents in mitochondria using an atomic absorption spectrophotometer (Hitachi type 207).

All glass and plastic wares used in this study were washed in conc HNO₃ beforehand. All values presented in the text and figures are mean ± SE. Significance test were calculated by Student's t-test taking P = 0.05 as the limit of significance.

RESULTS

Figure 1 shows the percentage changes in mean arterial blood pressure of each group and the representative ECG changes of noradrenaline infusion groups during the 5-hour infusion. Neither the blood pressure nor the ECG changed significantly in the control group. Although an initial increase in mean arterial blood pressure was observed in noradrenaline infusion groups 15 min after the infusion, a progressive dose-related decline was observed until the end of the experiment. About one hour after the infusion, diminution of QRS amplitude and ST segment elevation became apparent, and then Q wave abnormalities and T wave inversion developed in almost all cases. These hemodynamic and ECG changes were very similar to those reported by Moss et al indicating the progression of myocardial injury induced by the noradrenaline infusion.

The respiratory and oxidative phosphorylation activities of heart mitochondria in each group are shown in Table I. Fig. 2 provides representative profiles of oxidative phosphorylation from the control (A) and the group 2 (B) mitochondria. The control mitochondria were intact and tightly coupled as reflected by the high values of the rate of state 3 respiration, RCI and ADP/O ratio. These mitochondria demonstrated a mean rate of the ADP-stimulated oxygen consumption (state 3 respiration) of 270 ± 10 n atoms O/min/mg protein. In mitochondria prepared from group 1, state 3 respiration was slightly but significantly (p < 0.05) reduced. In group 2 mitochondria,
reduction in state 3 respiration became more prominent being $190 \pm 14$ n atoms O/min/mg protein in comparison to that of the control. RCI value decreased from $5.2 \pm 0.3$ of the control group to $4.3 \pm 0.2$ of group 2 ($p < 0.05$) whereas there were no significant difference in the ADP/O ratio and state 4 respiration among the three groups.

In one series of experiments, calcium binding and uptake activities of the mitochondria in the control and the noradrenaline groups were determined at different incubation intervals. Figs. 3 and 4 show time-courses of mitochondrial calcium binding and uptake activities, respectively, in the control group and in the group 2. There were no significant differences in the mitochondrial activity of calcium binding or in calcium uptake between these two groups. There were also no significant differences in the mitochondrial calcium transport activities between the control and group 1. These results show that noradrenaline-treated heart mitochondria can preserve calcium transport activity at a normal rate in our ATP added experimental condition even when the respiratory activity was markedly impaired. It was established that addition of sodium azide (5 mM), a well-known inhibitor of mitochondrial calcium transport, in these assay media produces about an 80% inhibition of both calcium binding and uptake activities. This finding was in accordance with the reports of other investigators, indicating that only a

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart mitochondrial calcium (nmol/mg protein)</th>
<th>Heart mitochondrial calcium (nmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Control (n = 8)</td>
<td>$9.1 \pm 0.6$</td>
<td>$17.5 \pm 1.3$</td>
</tr>
<tr>
<td>Group 1 (n = 8)</td>
<td>$12.8 \pm 0.8$ p &lt; 0.05</td>
<td>$17.3 \pm 1.2$ n.s.</td>
</tr>
<tr>
<td>Group 2 (n = 8)</td>
<td>$16.7 \pm 1.5$ p &lt; 0.005</td>
<td>$16.7 \pm 1.2$ n.s.</td>
</tr>
</tbody>
</table>

*Five-hour treatment with noradrenaline (Group 1: 2 μg/kg/min; Group 2: 5 μg/kg/min) caused a significant increase in the mitochondrial content of calcium in a dose-dependent manner. Mitochondrial content of magnesium was not affected significantly by noradrenaline treatment. All values are represented as mean ± SE of the number of samples in the parentheses. P values are calculated by using the non-paired t-test between the control and Group 1 or 2.*

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small and negligible amount of the microsomal fraction was contained in our mitochondrial samples.

Table II shows the contents of calcium and magnesium in heart mitochondria of each group. The calcium content in mitochondria significantly increased in a dose-dependent manner after the noradrenaline treatment from $9.1 \pm 0.6$ n moles/mg protein of the control to $12.8 \pm 0.8$ n moles/mg protein ($p < 0.05$) of group 1, and to $16.7 \pm 1.5$ n moles/mg protein ($p < 0.01$) of group 2. The magnesium content in mitochondria did not change significantly after the noradrenaline treatment.

**DISCUSSION**

It is well accepted that mitochondria are able to act as large capacity calcium reservoir, and to accumulate the excess calcium in the cytosol so that the concentration of calcium will not depart from the physiologically permissible range. Both the calcium concentration in the cytosol and the mitochondrial calcium transport activity are the major factors determining the mitochondrial calcium contents. Alterations in the mitochondrial ability to bind and uptake calcium have been reported in several pathologic conditions. In the present study, we found a significant increase in mitochondrial calcium content after noradrenaline treatment with no acceleration of mitochondrial calcium transport activity. These results suggest that the increase in mitochondrial calcium content is induced secondarily to the increase in cytosolic calcium concentration, which was caused by the enhancement of calcium influx into the myocardium exposed to high concentrations of noradrenaline. Therefore, the mechanism of intracellular calcium overload also appears to underlie the noradrenaline-induced myocardial damage similar to the case of isoproterenol.

The present study also demonstrated that noradrenaline-treated mitochondria showed significant decreases in the state 3 respiration rate and RCI value without a decrease in the ADP/O ratio. Recent studies have shown that there exists dissociation similar to that which we found in the present experiments between the state 3 respiration rate and RCI on the one hand and ADP/O ratio on the other under various experimental conditions, such as ischemic, ischemic reperfused and isoproterenol-treated hearts, when intracellular calcium overload is suggested. Sobel et al. however, have reported a depressed ADP/O ratio without significant changes of the state 3 respiration rate and the RCI value in noradrenaline-treated rats. These discrepancies in experimental results among investigators may be attributable to the differences in species, the method of producing cardiac injury and the mitochondrial isolation techniques. In any case, a defect in energy production leads to a failure to maintain the ATP-dependent mechanisms which normally operate to maintain intracellular homeostasis with respect to calcium. As a result, there will occur a pathologic increase in a cytosolic calcium concentration which would further inhibit the mitochondrial state 3 respiration in vivo. The understanding of this situation will be further complicated by the fact that mitochondria eventually will leak calcium into the cytosol. The consequences of a raised cytosolic calcium concentration include an ATP-wasting effect due to the activation of latent calcium-sensitive ATPases and a loss of membrane ultrastructure and integrity due to the activation of calcium-sensitive proteases and phospholipases. In this study the increase in mitochondrial calcium contents observed is not sufficient to explain the depression of the oxidative phosphorylation. Therefore, it is difficult to decide at the present time whether or not the decreased state 3 respiration actually resulted from so-called mitochondrial calcium overload. In this regard, other mechanisms such as the accumulation of longchain acyl-CoA induced by accelerated lipolysis by noradrenaline may also be possible causes. However, as pointed out by Wroge et al. it is also believed that the elevation of calcium content in mitochondria does not represent a uniform population and some mitochondria have excessively high calcium contents resulting in their functional and ultrastructural damages.

In conclusion, it was found that excessive noradrenaline causes intracellular calcium overload and the depression of mitochondrial respiration, and it is suggested that both of these changes play a key role in the pathogenesis of noradrenaline-induced myocardial injury through insufficient control of the cytosolic calcium level.

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