Influence of hypertension on cardiac contractile response of human erythrocyte-derived depressing factor in ventricular myocytes

Jun Ren\textsuperscript{a,b}, Yunyi Wen\textsuperscript{b} and Kadon K. Hintz\textsuperscript{c}

\textbf{Background} Erythrocyte-derived depressing factor (EDDF), a novel hypotensive factor purified from human erythrocytes, elicits endothelium-dependent vasorelaxation by reducing intracellular Ca\textsuperscript{2+} in vascular smooth muscle cells. However, its cardiac response is unknown.

\textbf{Objective} This study was designed to examine the cardiac contractile response of EDDF under both normotensive and hypertensive conditions.

\textbf{Methods} Ventricular myocytes were isolated from adult male spontaneously hypertensive rats (SHR) and age-matched Wistar–Kyoto (WKY) normotensive rats. Mechanical properties were evaluated using an IonOptix MyoCam system and intracellular Ca\textsuperscript{2+} was measured with fura-2 fluorescence. Myocytes were electrically stimulated to contract at 0.5 Hz. The contractile properties analyzed included peak shortening (PS), time-to-PS (TPS), time-to-90\% re-lengthening (TR\textsubscript{90}), maximal velocity of shortening/re-lengthening (\(\pm\) dl/dt), fura-fluorescence intensity change (\(\Delta F/F\)), and fura-fluorescence decay rate (r).

\textbf{Results} SHR rats displayed significantly elevated blood pressure. EDDF (10\textsuperscript{-9}–10\textsuperscript{-4} g/ml) did not affect PS, TPS, TR\textsubscript{90}, \(\Delta F/F\) and r but depressed \(\pm\) dl/dt at higher doses in WKY myocytes. However, EDDF depressed PS, \(\pm\) dl/dt and \(\Delta F/F\), shortened TPS without affecting TR\textsubscript{90} and r in SHR myocytes. Pretreatment of the myocytes with the nitric oxide synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) did not affect the EDDF-induced inhibition of PS and \(\pm\) dl/dt in SHR myocytes but unmasked an EDDF-induced negative response in WKY myocytes.

\textbf{Conclusions} These data indicate that EDDF may participate in the modulation of cardiac contractile function under hypertensive, but not normotensive, conditions. The cardiac depressive effect of EDDF is unlikely due to release of nitric oxide, as suggested in vascular smooth muscles. \textit{J Hypertens} 21:1183–1190 © 2003 Lippincott Williams & Wilkins.

\textbf{Introduction} Recently, a novel endogenous hypotensive agent was extracted from human erythrocytes in our laboratories and was defined as erythrocyte-derived depressing factor (EDDF) \cite{1,2}. EDDF significantly reduces blood pressure in both normotensive and experimental hypertensive animals including spontaneously hypertensive rats (SHR), stroke-prone SHR (SHR-sp), renal hypertensive rats \cite{1–4}. EDDF is rather safe with a LD\textsubscript{50} value of \(>1500\) mg/kg in rats \cite{5}. EDDF exists in human and other mammalian species such as pig, dog, rat, rabbit and cow, and has been found in many tissues including erythrocytes, brain, heart, liver and kidney, with the highest level found in erythrocytes \cite{2}. Studies from our group revealed that EDDF may elicit an endothelium-dependent vasorelaxation through accumulation of nitric oxide (NO), and subsequently, cGMP \cite{6,7}. This endothelium-dependent vasorelaxation is believed to be responsible, in large part, for the hypotensive property of EDDF. Our recent study indicated that EDDF may reduce cytosolic as well as nuclear Ca\textsuperscript{2+} in vascular smooth muscle cells (VSMCs) from human umbilical vein and Wistar rats \cite{2,4}. Further evidence suggested that EDDF may decrease DNA synthesis, inhibit agonist (angiotensin, norepinephrine)-induced cell proliferation, and diminish c-myc and calmodulin gene expression in VSMCs from SHR rats \cite{2}. The EDDF levels found in red blood cells were significantly reduced in patients and experimental animals with hypertension, compared with their normo-
tensive counterparts [2], suggesting that EDDF deficiency may contribute, at least in part, to the pathogenesis of hypertension.

Information on the relationship between EDDF and hypertension has been essentially limited to the vascular system. Little attempt has been made towards understanding the interaction between EDDF and cardiac contractile function under both normotensive and hypertensive conditions. Therefore, the aim of the present study was to evaluate the effect of EDDF on myocardial contraction and intracellular Ca\(^{2+}\) transients in isolated left ventricular myocytes from SHR rats and age-matched normotensive Wistar–Kyoto (WKY) rats. Due to the potential involvement of NO in EDDF-induced vascular response, the impact of NO synthase (NOS) inhibition on EDDF-induced cardiac response was also examined.

Materials and methods

**Purification of erythrocyte-derived depressing factor**

Whole blood was obtained from healthy volunteers of Peking Union Medical College Hospital, Beijing, China. The blood was centrifuged. The plasma and buffy coating were discarded. The packed erythrocytes were hemolyzed and the supernatant was subjected to gel column filtration by high-performance liquid chromatography (HPLC; Beckman System Cold Programmable Solvent Module 126; Beckman Instruments, Fullerton, California, USA). The following conditions were used for HPLC separation: column – C18 column (250 × 4 mm); mobile phase – acetic acid pH 7.0, Triethyl amine, acetonitrile; flow rate – 1.0 ml/min. Various fractions were tested for blood pressure regulation. The fraction containing inhibitory activity on blood pressure were preserved and used for this study [3,4,6].

**Experimental animals and isolation of ventricular myocytes**

The experimental procedures described here were approved by the animal investigation committees from University of North Dakota School of Medicine (Grand Forks, North Dakota, USA) and Peking Union Medical College (Beijing, China). Male Wistar–Kyoto (WKY) and SHR, 4 months of age, were obtained from Harlan Laboratories (Indianapolis, Indiana, USA) and maintained on a 12/12 h light/dark illumination cycle and ad libitum allowed food and water. The animals were provided by Harlan Teklad Laboratories (Indianapolis, Indiana, USA) and main- tained on a 12/12 h light/dark illumination cycle and ad libitum allowed food and water. SHR, 4 months of age, were obtained from Harlan Technologies (Indianapolis, Indiana, USA) and main- tained on a 12/12 h light/dark illumination cycle and ad libitum allowed food and water. SHR, 4 months of age, were obtained from Harlan Teklad Laboratories (Indianapolis, Indiana, USA) and main- tained on a 12/12 h light/dark illumination cycle and ad libitum allowed food and water.

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emission. Since intracellular Ca\(^{2+}\) transients in cardiac myocytes are short-lived with a fast kinetics, the ‘interpolated’ method offers better signal-to-noise ratio than the ‘interleaved’ method (rapidly switching between the 340 and 380 nm excitation wavelengths). Myocytes were discarded if the 360 signal displayed any decay > 10%.

**Experimental protocols**

Myocytes (either fura-2 loaded or non-loaded) were first allowed to contract at a stimulation frequency of 0.5 Hz for 10 min to ensure steady state (myocytes with rundown > 10% were not studied further) before superfusing with EDDF (10\(^{-9}\)–10\(^{-4}\) g/ml). EDDF was extracted and purified from human erythrocytes using blood obtained from healthy volunteers offered by the blood bank of Peking Union Medical College Hospital (Beijing, China). Cells were then washed with normal contractile buffer for 5 min. In some studies, \(\text{N^o-nitro-L-arginine methyl ester (L-NAME, 100 \mu M)}\) was incubated with the myocytes for 10 min prior to EDDF addition.

**Data analysis**

For each experimental series, data are presented as Mean ± SEM. Statistical significance (\(P < 0.05\)) for each variable was estimated by two-way analysis of variance (ANOVA) or \(t\)-test, where appropriate.

**Results**

**Experiment animals**

The SHR rats exhibited significantly elevated blood pressure and enhanced body weight compared with WKY counterparts. The heart and kidney weights were similar for the two groups although liver weight was significantly lower in SHR rats compared with WKY rats (Table 1).

**Mechanical and fluorescent properties of WKY and SHR myocytes**

The average resting cell length (CL) for myocytes used in this study was 154.2 ± 3.2 μm (\(n = 35\) myocytes) and 160.5 ± 5.4 μm (\(n = 38\) myocytes) (\(P < 0.05\)), in WKY and SHR groups, respectively. However, the cross-sectional area of myocytes was significantly larger in SHR myocytes (8245 ± 374 μm\(^2\), \(n = 38\)) than that of the WKY group (6437 ± 268 μm\(^2\), \(n = 35\)). Peak shortening (PS) was not significantly different between the WKY (6.0 ± 0.7% CL) and the SHR (4.9 ± 0.6% CL) groups. Myocytes from both groups displayed similar TPS, TR\(_{90}\), and \(\text{dl/dt}\) (Table 2, baseline value). Furthermore, the intracellular Ca\(^{2+}\) transient fluorescent measurement revealed that the resting intracellular Ca\(^{2+}\) levels (360/380 ratio) were similar between the two groups whereas the intracellular Ca\(^{2+}\) clearing rate (\(\tau\)) was significantly higher in myocytes from SHR rats compared with those from WKY rats, indicating impaired intracellular Ca\(^{2+}\) clearing under hypertension (Table 3).

**Table 1** General features of 25 week-old WKY and SHR rats

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Body weight (g)</th>
<th>Heart weight (g)</th>
<th>Heart weight/body weight (mg/g)</th>
<th>Liver weight (g)</th>
<th>Kidney weight (g)</th>
<th>Systolic blood pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (6)</td>
<td>389 ± 11</td>
<td>1.98 ± 0.06</td>
<td>5.08 ± 0.12</td>
<td>13.0 ± 0.5</td>
<td>2.66 ± 0.11</td>
<td>121 ± 2</td>
</tr>
<tr>
<td>SHR (6)</td>
<td>429 ± 12*</td>
<td>1.92 ± 0.11</td>
<td>4.49 ± 0.25*</td>
<td>11.8 ± 0.3*</td>
<td>2.65 ± 0.08</td>
<td>188 ± 3*</td>
</tr>
</tbody>
</table>

Data represents mean ± SEM; \(n\) is presented in parentheses. *\(P < 0.05\) versus WKY group. WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats.

**Table 2** Effect of EDDF on mechanical characteristics (percentage change from respective baseline value) in cells from adult WKY and SHR rat hearts

<table>
<thead>
<tr>
<th>Time-to-peak shortening (TPS)</th>
<th>Time-to-90% re-lengthening (TR(_{90}))</th>
<th>Maximal velocities of shortening ((\text{dl/dt}))</th>
<th>Maximal velocities of re-lengthening ((\text{dl/dt}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (Baseline)</td>
<td>239 ± 14</td>
<td>336 ± 30</td>
<td>66.4 ± 9.7</td>
</tr>
<tr>
<td>EDDF 10(^{-9}) g/ml</td>
<td>1.32 ± 2.86</td>
<td>−5.48 ± 3.71</td>
<td>9.28 ± 5.94</td>
</tr>
<tr>
<td>EDDF 10(^{-8}) g/ml</td>
<td>−0.74 ± 4.03</td>
<td>3.06 ± 7.92</td>
<td>11.04 ± 9.87</td>
</tr>
<tr>
<td>EDDF 10(^{-7}) g/ml</td>
<td>7.81 ± 4.71</td>
<td>−5.38 ± 4.57</td>
<td>−4.99 ± 6.95</td>
</tr>
<tr>
<td>EDDF 10(^{-6}) g/ml</td>
<td>1.47 ± 2.08</td>
<td>−0.66 ± 6.16</td>
<td>−7.32 ± 8.55</td>
</tr>
<tr>
<td>EDDF 10(^{-5}) g/ml</td>
<td>3.69 ± 3.93</td>
<td>−3.01 ± 6.34</td>
<td>−13.84 ± 9.66</td>
</tr>
<tr>
<td>EDDF 10(^{-4}) g/ml</td>
<td>−0.16 ± 4.52</td>
<td>0.09 ± 6.70</td>
<td>−16.59 ± 6.65</td>
</tr>
<tr>
<td>SHR (Baseline)</td>
<td>189 ± 33</td>
<td>31.1 ± 66</td>
<td>69.8 ± 8.2</td>
</tr>
<tr>
<td>EDDF 10(^{-9}) g/ml</td>
<td>−6.51 ± 3.41</td>
<td>9.37 ± 8.28</td>
<td>−9.76 ± 6.64</td>
</tr>
<tr>
<td>EDDF 10(^{-8}) g/ml</td>
<td>−0.13 ± 5.78</td>
<td>−1.34 ± 7.77</td>
<td>−23.37 ± 6.56</td>
</tr>
<tr>
<td>EDDF 10(^{-7}) g/ml</td>
<td>−8.70 ± 5.15</td>
<td>−2.37 ± 6.72</td>
<td>−17.64 ± 9.27</td>
</tr>
<tr>
<td>EDDF 10(^{-6}) g/ml</td>
<td>−11.99 ± 5.41</td>
<td>3.97 ± 6.62</td>
<td>−25.40 ± 10.24</td>
</tr>
<tr>
<td>EDDF 10(^{-5}) g/ml</td>
<td>−8.29 ± 4.42</td>
<td>−6.47 ± 6.40</td>
<td>−24.58 ± 10.82</td>
</tr>
<tr>
<td>EDDF 10(^{-4}) g/ml</td>
<td>−9.67 ± 4.80</td>
<td>−7.39 ± 5.17</td>
<td>−25.80 ± 12.51</td>
</tr>
</tbody>
</table>

Data represents percentage change from respective baseline value. TPS, Time-to-peak shortening; TR\(_{90}\), time-to-90% re-lengthening; \(\text{dl/dt}\), maximal velocities of shortening and re-lengthening. Mean ± SEM. *\(P < 0.05\) versus respective baseline. \(n = 22\) and 28 cells for Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR), respectively. EDDF, erythrocyte-derived depressor factor.
Effect of EDDF on myocyte shortening

Acute exposure (up to 15 min) of EDDF did not alter the resting cell length over the range of concentrations tested ($10^{-9} - 10^{-4}$ g/ml). A representative trace depicting the effect of EDDF ($10^{-7}$ g/ml) on myocyte shortening is shown in Figure 1a. At the end of a 5-min exposure to this dose of EDDF, PS was not affected in WKY myocytes. However, PS was decreased by about 25% in SHR myocytes. Data in Figure 1b shows that EDDF produced a dose-dependent depression of myocyte shortening in cells from SHR, but not WKY rats with a maximal inhibition of 40.3%. The depressive

Table 3 Effect of EDDF on intracellular Ca$^{2+}$ transient properties in myocytes from adult WKY and SHR rat hearts

<table>
<thead>
<tr>
<th></th>
<th>Resting intracellular Ca$^{2+}$ (360:380 ratio)</th>
<th>Intracellular Ca$^{2+}$ decay rate ($r$, ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (Control)</td>
<td>1.056 ± 0.026</td>
<td>179 ± 5</td>
</tr>
<tr>
<td>EDDF 10^{-7} (mg/ml)</td>
<td>1.045 ± 0.014</td>
<td>192 ± 14</td>
</tr>
<tr>
<td>EDDF 10^{-6} (mg/ml)</td>
<td>1.027 ± 0.018</td>
<td>205 ± 13</td>
</tr>
<tr>
<td>SHR (Control)</td>
<td>1.032 ± 0.030</td>
<td>287 ± 43*</td>
</tr>
<tr>
<td>EDDF 10^{-7} (mg/ml)</td>
<td>1.035 ± 0.028</td>
<td>298 ± 66</td>
</tr>
<tr>
<td>EDDF 10^{-6} (mg/ml)</td>
<td>1.016 ± 0.025</td>
<td>326 ± 59</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM. *$P < 0.05$ versus baseline value from Wistar–Kyoto (WKY) group, $n = 10$ myocytes for WKY and spontaneously hypertensive (SHR) groups. EDDF, erythrocyte-derived depressing factor.

Fig. 1

Typical experiment showing the effect of erythrocyte-derived depressing factor (EDDF) ($10^{-7}$ g/ml) on myocyte shortening in ventricular myocytes isolated from (a) adult male WKY and (b) SHR rat hearts. Myocyte shortening and re-lengthening were recorded at 25°C before (solid line) and 5 min after (dashed line) EDDF administration. (c) Concentration-dependent response to EDDF ($10^{-9} - 10^{-4}$ g/ml) on myocytes shortening (PS) from Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rat hearts. Peak shortening (PS) is expressed as the percentage change of respective control value. Mean ± SEM, sample size is given in parentheses, *$P < 0.05$ versus control value, †$P < 0.05$ versus respective from WKY group.
The effect of EDDF on cell shortening was maximal within 5 min of exposure and was not reversible upon washout (data not shown). The durations of myocyte shortening and re-lengthening (TPS and TR90) were not affected by EDDF in myocytes from WKY rats. However, EDDF shortened TPS without affecting TR90 in myocytes from the SHR group. The maximal velocities of shortening and re-lengthening ($\pm d/dt$) were significantly reduced by high doses of EDDF ($10^{-3}$ and $10^{-4}$ g/ml) in WKY myocytes. Interestingly, SHR myocytes were more prone to EDDF-induced inhibition on $\pm d/dt$ with a threshold lower than $10^{-9}$ g/ml (Table 2).

**Effect of EDDF on intracellular Ca$^{2+}$ transients**

To determine whether the EDDF-induced response in myocyte shortening was due to changes in availability of intracellular free Ca$^{2+}$ ([Ca$^{2+}]_i$), we examined $[Ca^{2+}]_i$ in response to electrical stimuli in the presence of EDDF ($10^{-7} - 10^{-6}$ g/ml). Representative traces of intracellular Ca$^{2+}$ transients shown in Figure 2 depicts...
that $10^{-7}$ and $10^{-6}$ g/ml EDDF had no significant effect on AFFI in WKY myocytes whereas it elicited a $\sim 21$ and $\sim 29\%$ inhibition of AFFI, respectively, in SHR myocytes. The inhibition of AFFI suggests that a decrease in intracellular free Ca$^{2+}$ recruitment may be responsible for EDDF-induced depressive action on myocyte shortening. Neither resting FFI (representing resting Ca$^{2+}$ level) nor $\tau$ (fluorescence decay time) was significantly altered by EDDF in both groups (Table 3).

**Effect of EDDF on PS in the presence of L-NAME**

EDDF-induced vasorelaxation has been demonstrated to be associated with nitric oxide (NO) release [7]. Constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) are both present in cardiac myocytes [9]. To examine if NO is involved in the action of EDDF in WKY and SHR myocytes, the effect of EDDF on PS was re-examined in the presence of the NOS inhibitor L-NAME (100 µmol/l). L-NAME alone had no effect on PS over 30 min (data not shown). As shown in Figure 3, the EDDF-induced decrease in PS in SHR myocytes was not affected by L-NAME pretreatment. Consistently, the EDDF-induced inhibition of $\pm dF/dt$ was not altered by L-NAME pretreatment in SHR myocytes (data not shown). Interestingly, L-NAME unmasked an EDDF-induced negative response in WKY myocytes when a high dose of EDDF ($10^{-6}$ g/ml) was applied. These data suggest that the EDDF-induced cardiac depression is unlikely to be associated with NO production.

**Discussion**

This is the first study examining the cardiac contractile effect of EDDF and influence of hypertension on EDDF-induced cardiac contractile response in isolated ventricular myocytes. Our data revealed that while EDDF exhibits minimal effect on myocyte mechanics and intracellular Ca$^{2+}$ handling in normotensive WKY rats (only $\pm dF/dt$ was depressed at high EDDF levels), it depresses cardiac contraction (PS and $\pm dF/dt$) and intracellular Ca$^{2+}$ rise (AFFI) associated with shortened duration of contraction (TPS) and normal duration of relaxation ($T_{R90}$) in spontaneously hypertensive SHR rats. The non-selective NOS inhibitor L-NAME did not affect the EDDF-induced cardiac responses in SHR myocytes whereas it unmasked a negative PS response of EDDF in WKY myocytes. Collectively, these results suggest that EDDF may not be a major factor in the regulation of cardiac contractile function under normotensive condition. However, it appears to play a role in the modulation of cardiac contractile function through NO-independent mechanisms under hypertensive condition.

In our study, the SHR rats displayed a significantly elevated blood pressure and body weight associated with hepatic atrophy compared with the WKY group. During the sustained course of hypertension, several pathological changes may occur in the liver including hepatic parenchymal atrophy, portal and parenchymal fibrosis, portal venous phlebosclerosis and progressively deteriorated hepatic function [10]. These morphological as well as enzymatic changes may be related to hypertension-induced neurohormonal alterations such as accumulation of substance P and catecholamines in the liver, leading to decompensated hepatic function in patients with hypertension [11]. The decrease in heart-to-body weight ratio is essentially due to the significantly enhanced body weight as the absolute heart weights are comparable in WKY and SHR rats.

EDDF is a novel endogenous hypotensive factor and induces a significant depressing effect on blood pressure. It is safe and present widely in humans and other animals. Although the blood levels of EDDF have not been directly measured, approximately 10–100 mg purified EDDF may be obtained from every liter of whole blood from healthy human or pig, which would be equivalent to a EDDF levels of $10^{-5}$ to $10^{-4}$ g/ml (Wen et al., unpublished data). Our earlier studies have indicated that EDDF is rather unique from any other known endogenous vasoactive substances and may be a potential new endogenous candidate for blood pressure regulation. The fact that the levels of EDDF in erythrocytes from hypertensive human and experimen-
The cardiac contractile function under normotensive conditions, the underlying mechanisms of the EDDF-induced cardiac contractile response under hypertensive condition are largely pending on the successful identification of the EDDF. NO has been found to exert both a positive (at low dose) and a negative (at high dose) cardiac contractile effect [9,16,17]. Although our findings with L-NAME do not favor any NO-mediated pathway in the EDDF-induced cardiac depressant response, caution needs to be taken regarding the causal relationship between NO and EDDF-induced cardiac response. NOS activity is enhanced under genetic SHR hypertension [18]. However, this increase is ‘effectively’ cancelled by an increased inactivation of NO under hypertension [19]. The elevated NO inactivation in SHR may abrogate potential effect of EDDF on the NO system under hypertensive conditions, although it is not certain whether the facilitated NO inactivation due to genetic hypertension contributes to the disparate cardiac contractile responses to EDDF under normotensive and hypertensive conditions seen in the current experiment.

In conclusion, although the present study has shed some light on the role of EDDF in cardiovascular function under both normotensive and hypertensive conditions, the underlying mechanisms of the EDDF-induced cardiac and vascular response is far from being complete. Not surprisingly, further study of EDDF is largely pending on the successful identification of the chemical structure and optimization of the purification procedures of EDDF.

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References


