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Leptin suppresses non-apoptotic cell death in ischemic rat cardiomyocytes by reduction of $iPLA_2$ activity

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ABSTRACT

Caspase-independent, non-apoptotic cell death is an important therapeutic target in myocardial ischemia. Leptin, an adipose-derived hormone, is known to exhibit cytoprotective effects on the ischemic heart, but the mechanisms are poorly understood. In this research, we found that pretreatment of leptin strongly suppressed ischemic-augmented nuclear shrinkage and non-apoptotic cell death on cardiomyocytes. Leptin was also shown to significantly inhibit the activity of $iPLA_2$, which is considered to play crucial roles in non-apoptotic cell death, resulting in effective prevention of ischemia-induced myocyte death. These findings provide the first evidence of a protective mechanism of leptin against ischemia-induced non-apoptotic cardiomyocyte death.

1. Introduction

Myocardial ischemia causes depressed myocardial functions and associated deleterious morphological alterations that lead to serious heart failure. Ischemic injury is induced in a variety of forms of apoptosis and necrosis [1,2]. Although many parts of apoptotic molecular mechanisms have already been elucidated, ischemia-induced non-apoptotic myocyte death is not clearly defined [3,4]. However, we recently found that non-apoptotic cell death is another crucial form of regulated cell death in ischemia-exposed cardiac myocytes [5], and we emphasize the importance of investigation for non-apoptotic forms as well as apoptosis to fully understand the spectrum of death in myocardial ischemia.

An obesity gene product, leptin, is a 16-kDa adipose hormone that has been shown to protect the myocardium against ischemia–reperfusion injury [6–8]. In the elucidated molecular mechanisms, leptin activates the reperfusion injury salvage kinase (RISK) pathway through phosphatidylinositol 3-kinase (PI3–K)-cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase, and mediates JAK/STAT signaling linked to the mitochondrial permeability transition pore (MPTP) [6]. In addition, leptin-mediated cardiomyocyte protection was recently reported to inhibit apoptosis by increasing antioxidant defense, which blocks the mitochondrial-dependent intrinsic apoptotic pathway [9], and by activating signal transducer and activator of transcription (STAT)-3-responsive anti-apoptotic genes, including B-cell lymphoma (Bcl)-2 and survivin, which serve to downregulate the activity of caspase-3 [10]. Moreover, previous studies showed that pretreatment with leptin attenuated H_2O_2-induced apoptosis in H9c2 cells [11]. Pretreatment with leptin also protected cultured myocytes from hypoxic damage [12]. The authors suggest that leptin successfully inhibits apoptosis at comparatively low concentrations (10–100 nM) in their reports. However, the mechanisms of action and potency of leptin in ischemic cardiomyocytes as a cardioprotector against non-apoptotic cell death have not yet been assessed in detail, and its evaluation is strongly required to develop sophisticated strategies for effective cardioprotection for future therapy [7].

In the present study, we investigated the biological activity of leptin against non-apoptotic cell death under conditions of oxygen, glucose, and serum deprivation, which mimic myocardial ischemia, and leptin showed significant suppression of ischemia-induced, non-apoptotic myocyte death by decreasing $iPLA_2$ activity, which is considered to play crucial roles in the process of non-apoptotic cell death.

Abbreviations: BSA, bovine serum albumin; CRH, corticotropin-releasing hormone; DMEM, Dulbecco's modified Eagle's medium; $iPLA_2$, calcium-independent phospholipase A_2; BEL, bromoenol lactone; LDH, lactate dehydrogenase; PI, propidium iodide.

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2. Materials and methods

All of the experimental procedures *in vivo* were approved by the review board of the Institutional Animal Care Committee of Mukogawa Women's University.

2.1. Primary culture of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiac myocytes from 1- to 2-day-old Wistar rats (Japan SLC, Hamamatsu, Japan) were prepared according to a previously described procedure [5,13–15]. This procedure yielded cell preparations containing 90–95% myocytes, as assessed by microscopic observation of cell beating (data not shown). The myocytes were seeded in 96-, 48-, or 24-well microplates at a density ~80% confluence and were cultured for 3 days in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, USA) containing 5% defined newborn calf serum (Invitrogen), 3 mM pyruvic acid (Nacalai Tesque, Kyoto, Japan), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium (Roche Diagnostics, Mannheim, Germany), and 100 μM 5-bromo-2-deoxyuridine (Nacalai Tesque) to prevent the proliferation of non-myocytes. The culture medium was replaced daily.

2.2. Ischemia model: oxygen, glucose, and serum deprivation

Ischemia model were prepared according to a previously described procedure [5]. The culture medium was replaced to glucose- and serum-free DMEM, and the cells were exposed to an anaerobic environment prepared using an Anaeropack system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37 °C for 6 h. During this period, the concentration of O_2 in the medium was 0.02% and that of CO_2 was 5%. Leptin from rat (Sigma, St. Louis, MO, USA) was added to the myocyte culture medium for 1 h prior to ischemic stimulation. As a control, cells were incubated under normoxic conditions at 37 °C in culture medium, which was equilibrated with a 5% CO_2–95% air atmosphere.

2.3. Evaluation of ischemia-induced cell death

Ischemic damage in the myocytes was quantified by measuring the release of lactate dehydrogenase (LDH) into the culture medium using a CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA). To determine cell death patterns, cells were stained by incubation with propidium iodide (PI; 2 μg/mL) and a fluorescent conjugate of Annexin V (×1/20; Vybrant Apoptosis Assay Kit; Molecular Probes, Inc., Eugene, OR, USA) or Hoechst 33342 dye (50 μM). Fluorescence images were captured using a fluorescent microscopy system (Nikon, Tokyo, Japan) and the area of each nucleus was measured using LuminaVision software (Mitani Corp., Tokyo, Japan). Cardiomyocytes exposed to ischemia in the presence of glucose and serum for 24 h were used as the positive control [5].

2.4. Measurement of $iPLA_2$ activity

The $iPLA_2$ activity in myocytes was measured using a modified commercial assay kit that was originally designed for cytosolic phospholipase A_2 ($cPLA_2$) (Cayman Chemical Co., Ann Arbor, MI, USA) as previously described [5].

2.5. Statistical analysis

All results are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism software (ver. 5.00; GraphPad, San Diego, CA, USA). One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. Differences were considered significant when the calculated p value was <0.05.

3. Results and discussion

3.1. Leptin prevents ischemia-induced, non-apoptotic myocyte death

In agreement with our previous finding [5], caspase-independent, non-apoptotic myocyte death was induced by ischemia in glucose- and serum-deficient medium. After a 6 h ischemic insult, a significant fraction of cells died, and the pan-caspase inhibitor zVAD-fmk, which prevents all caspase-dependent apoptotic pathways, did not inhibit this cell death analyzed by LDH release assay, demonstrating low dependency of caspases in the process of ischemic cell death in this experimental model (Fig. 1). In ischemic model under the glucose- and serum-containing medium, apoptotic cell death were strongly prevented by zVAD-fmk in the same experimental condition [5]. Fig. 2A shows representative fluorescence microscopic observations of PI and Annexin V staining of myocytes after a 6 h ischemic insult to detect disruption of cellular membranes and cell death (PI), and apoptotic membrane organization (Annexin V). The ischemia-exposed cells were positive for PI and negative for Annexin V in the presence or absence of zVAD-fmk, indicating necrotic loss of membrane integrity, but not apoptotic cell death. The dead cells exhibited PI-positive and Annexin V-negative staining was reported to indicate non-apoptotic cell death in cardiomyocytes [16] and any caspase-3 activity and DNA ladder under ischemia in the glucose- and serum-deficient medium were not detected as previously reported [5].

To investigate whether pretreatment with leptin inhibits ischemic damage by preventing non-apoptotic myocyte death, the effects against cell death were assessed by LDH release experiment. Pretreatment with leptin (6 nM) for 1 or 6 h, but not 24 h, before ischemic stimulation for 6 h significantly inhibited cell death, whereas the cells exposed to ischemia even in the presence of zVAD-fmk resulted in significant cell death (Fig. 1). Pretreatment

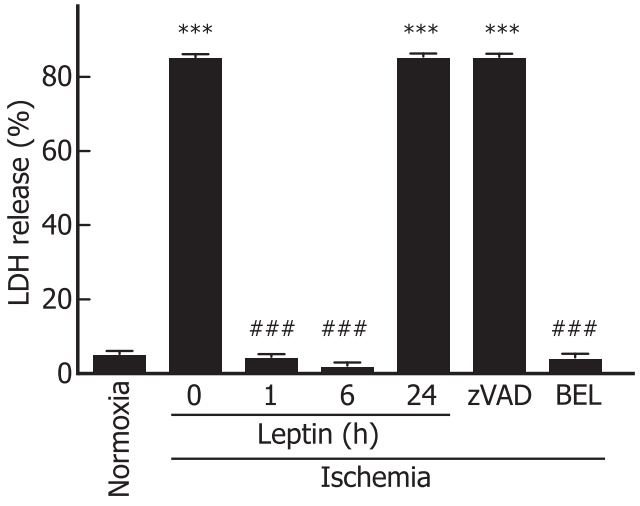

Fig. 1. Leptin protects cultured neonatal rat cardiomyocytes from ischemia-induced cell death. Myocytes were treated in the absence or presence of leptin (6 nM) for 1, 6, or 24 h prior to exposure to ischemia in glucose- and serum-deficient medium. After 6 h of ischemia treatment, cell death was measured by LDH release assay. BEL (40 μM) or zVAD-fmk (100 μM) was added to the glucose- and serum-deficient medium during ischemia. Data represent the mean ± SEM of three independent experiments. ***, $p < 0.001$ versus normoxia; ###, $p < 0.001$ versus ischemia.

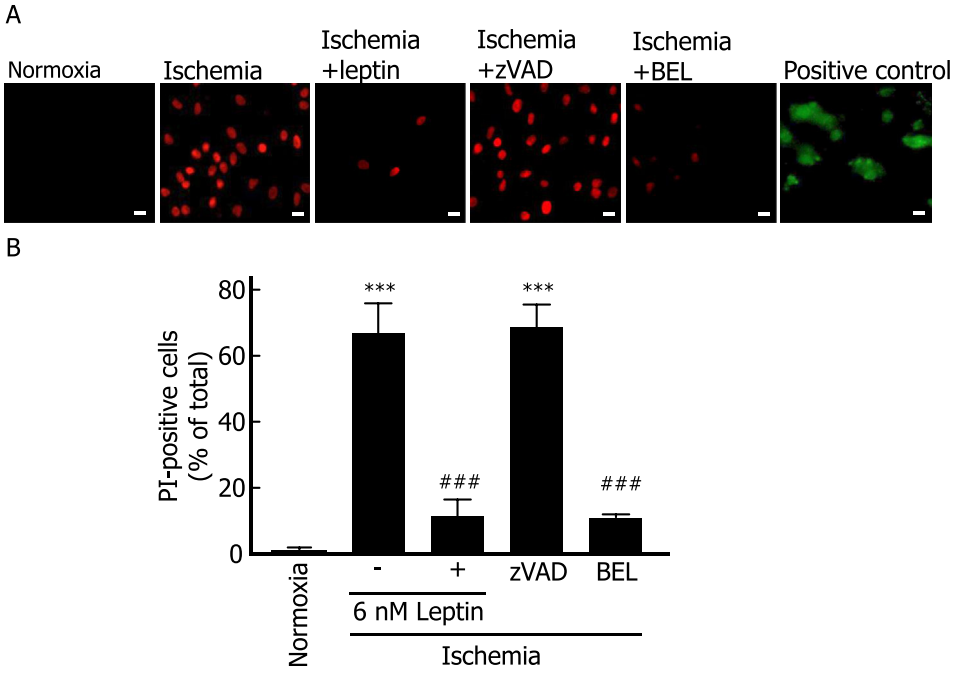

Fig. 2. Effect of leptin on non-apoptotic cardiomyocyte death. Myocytes were pretreated in the absence or presence of leptin (6 nM) for 1 h and then exposed to ischemia for 6 h in glucose- and serum-deficient condition. BEL (40 μM) or zVAD-fmk (100 μM) was also added in this experimental condition during ischemia. (A) Myocytes were stained with PI and fluorescently-labeled Annexin V. Cardiomyocytes exposed to ischemia in the presence of glucose and serum for 24 h were used as the positive control. Representative results of three independent experiments are shown. Scale bars represent 10 μm. (B) The percentage of PI-positive cells is given. Samples (n = 222–1404) were obtained from three different primary culture preparations. Data represent the mean ± SEM. ***, $p < 0.001$ versus normoxia; ###, $p < 0.001$ versus ischemia.

with leptin did not affect Annexin V staining (Fig. 2A) but significantly reduced the proportion of ischemia-exposed dead cells that were positive for PI from 67% to 11% (Fig. 2B). These data suggest that isolated neonatal cardiomyocytes showed resistance to ischemia-induced, caspase-independent cell death when pretreated with leptin in a low concentration. However, long (24 h) treatment of leptin did not prevent the cell death, because treatment with leptin in a long time was shown to induce their lipid accumulations, leading to loss of leptin activity [11,17,18].

In vivo, leptin was shown to reduce the infarct size during reperfusion in perfused hearts [6,8]. Although apoptosis may contribute to some extent to the exacerbation of myocardial injury, the major type of cardiomyocyte death induced by ischemic stimulation or ischemia–reperfusion is considered to be non-apoptotic cell death [1,19]. Leptin plays an important role in protecting against ischemia-induced myocyte death as well as apoptosis, and our findings will be essential for the development of novel therapeutic strategies to promote cell survival following myocardial ischemia.

3.2. Leptin suppresses nuclear shrinkage and iPLA$_2$ activity in non-apoptotic myocyte death

Next, we investigated the cardioprotective effect of leptin on nuclear shrinkage in ischemic, caspase-independent cell death. In our previous study, morphological change of the nuclear shrinkage was shown without chromatin fragmentation in the cell death [20] after ischemia induction in glucose- and serum-deficient condition in *in vitro* model [5]. Apoptotic nuclear changes, such as chromatin condensation and fragmentation were completely prevented by 100 μM zVAD-fmk, whereas zVAD-fmk had no effect on the nuclear shrinkage in the oxygen-, glucose- and serum-deficient condition [5]. In Fig. 3, nuclear area measurements were detected to assess the effects of leptin on ischemia-augmented nuclear shrinkage. After 6 h of ischemic stimulation, in glucose- and serum-deficient condition, the nuclear area was significantly reduced from 189 to 43 μm^2. Myocytes subjected ischemic stimulation for 24 h also showed nuclear shrinkage without chromatin fragmentation [5]. On the other hand, nuclear shrinkage was effectively prevented in cells pretreated with leptin (6 nM) and same ischemic condition, whose nuclear areas (131 μm^2) were similar to those of cells maintained under normoxic conditions.

We previously reported that iPLA$_2$ play crucial roles in induction of caspase-independent, non-apoptotic myocyte death by ischemia in glucose- and serum-deficient condition [5]. Ischemia-induced cell death was strongly inhibited by the iPLA$_2$ inhibitor, bromoenol lactone (BEL) (Figs. 1 and 2). Moreover, treatment with BEL prevented nuclear shrinkage in myocytes exposed to ischemia (Fig. 3). Therefore, we examined whether the leptin also modulates iPLA$_2$ activity in caspase-independent cell death. As shown in Fig. 4, ischemia led to a substantial increase in iPLA$_2$ activity in the presence or absence of zVAD-fmk. Surprisingly, pretreatment with leptin (6 nM) for 1 h reduced iPLA$_2$ activity by ~50% in comparison to untreated ischemic cardiomyocytes. The iPLA$_2$ activity in basal level is very low and there is no change by Leptin or BEL (data not shown). These results demonstrate that the cardioprotective effects of the leptin were accompanied by the suppression of iPLA$_2$ activity under ischemic conditions.

Leptin is though to inhibit the ischemic injury through cPLA$_2$, however not cPLA$_2$ but iPLA$_2$ targets non-apoptotic cell death. Because iPLA$_2$ in PLA$_2$ family is crucial for a ischemic caspase-independent, non-apoptotic cell death pathway [20]. Previously, we demonstrated that corticotropin-releasing hormone and urocortin suppress ischemia-induced, non-apoptotic cardiomyocyte death by reducing the expression of iPLA$_2$ [5]. A similar mechanism may be involved in the cardioprotective effect of leptin because leptin has been reported to activate a PKA signaling pathway downstream of corticotropin-releasing factor type 2 receptor in the

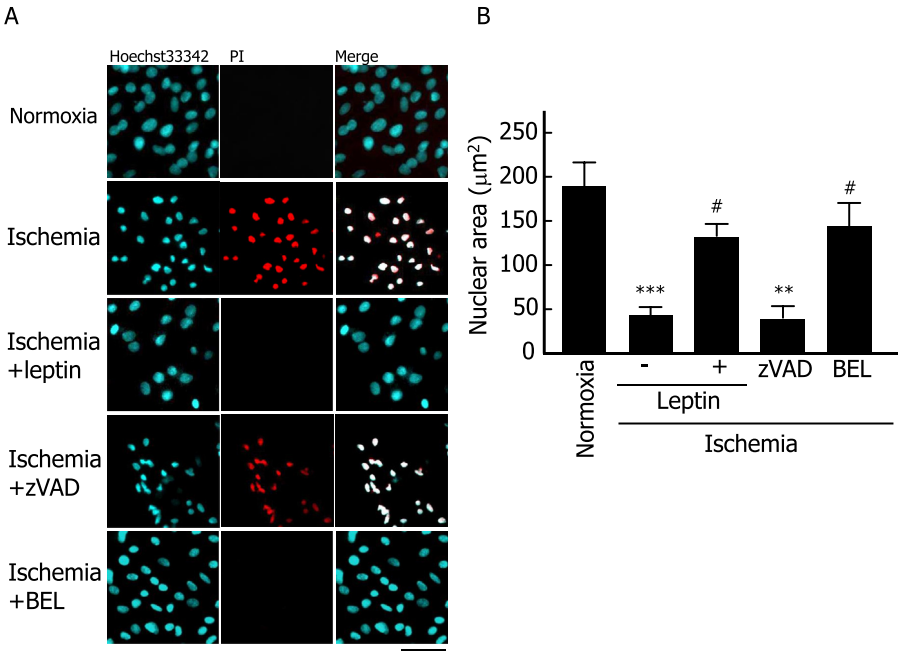

Fig. 3. Leptin suppresses nuclear shrinkage induced in a caspase-independent manner. Myocytes were pretreated in the absence or presence of leptin (6 nM) for 1 h and then exposed to ischemia for 6 h in glucose- and serum-deficient condition. BEL (40 μM) or zVAD-fmk (100 μM) was added in same experimental condition during ischemia. (A) Nuclei were stained with Hoechst 33342. Representative results of three independent experiments are shown. Scale bars represent 50 μm. (B) The areas of shrunken nuclei were individually determined from the fluorescence microscopy images. Samples (n = 222–1069) were obtained from three different primary culture preparations. Data represent the mean ± SEM. ***, $p < 0.001$ versus normoxia; #, $p < 0.001$ versus ischemia.

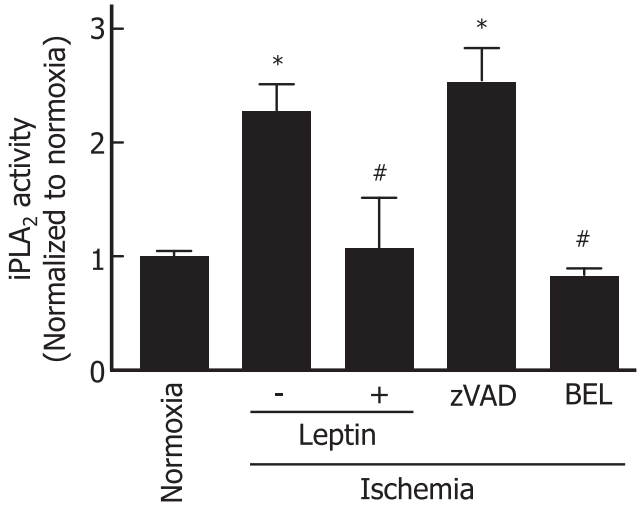

Fig. 4. Leptin reduces ischemia-induced $iPLA_2$ activity in a caspase-independent manner. Myocytes were pretreated in the absence or presence of leptin (6 nM) for 1 h and then exposed to ischemia for 6 h in glucose- and serum-deficient condition in the presence or absence of BEL (40 μM) and zVAD-fmk (100 μM). Data represent the mean ± SEM of three independent experiments. *, $p < 0.05$ versus normoxia; #, $p < 0.05$ versus ischemia.

heart, which is considered to regulate $iPLA_2$ activity [21,22]. Moreover, Zheng et al. reported that leptin protects cardiomyocytes from serum deprivation-induced apoptosis by activating superoxide dismutase [9]. Reactive oxygen species were shown to be produced in $iPLA_2$-dependent cell death under hypoxia/low glucose conditions [23]. Although further studies are needed, leptin may induce cardioprotection against non-apoptotic cell death by increasing antioxidant activity. Leptin has also been shown to increase nitric oxide synthetase (NOS) activity [24]. Ischemic preconditioning increases NOS activation in the heart, contributing to protection against ischemic damage [25]. Therefore, a preconditioning effect might be one of the underlying reasons for cardioprotection against ischemia by leptin, and further studies should be needed for future therapy. Recently the cardiac-specific leptin receptor knock-out (ObRKO) mice have been generated by McGaffin et al. and demonstrated to exacerbate ischemic heart failure through STAT3 and 5′-Adenosine monophosphate-activated protein kinase (AMPK) pathway [26]. Although the suppression of ischemic injury is not clarified whether to inhibition inflammation or cell death directly, it is possible that the analysis of ObRKO mice is clear the role of leptin signal pathway in non-apoptotic cell death.

In conclusion, our findings show that leptin effectively suppresses ischemia-induced, non-apoptotic myocyte death by inhibiting $iPLA_2$ activation. This is the first study to demonstrate the molecular mechanisms of the anti-non-apoptotic effects of leptin against ischemic cardiomyocytes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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