Neutrophil Gelatinase-Associated Lipocalin2 Exaggerates Cardiomyocyte Hypoxia Injury by Inhibiting Integrin β3 Signaling

Background: The neutrophil inflammatory protein, lipocalin-2 (NGAL), is elevated in certain forms of cardiac hypertrophy and acute heart failure. However, the specific role of NGAL in cardiac hypoxia injury is unclear. This study aimed to elucidate the functional role of NGAL in cardiomyocyte hypoxia injury.

Material/Methods: Neonatal rat cardiomyocytes were transfected with adenovirus [Ad-NGAL] to overexpress human-NGAL and then were exposed to hypoxia for 24 h to establish a hypoxia model. Cell inflammation was detected by RT-PCT and ELISA assay. Cell apoptosis was detected by TUNEL assay. Oxidative stress was also detected by commercial kits.

Results: An increased inflammatory response, apoptosis, and augmented oxidative stress were observed after exposure to hypoxia, while NGAL overexpression in cells increased the expression and release of inflammatory cytokines. NGAL overexpression also increased the number of apoptotic cells and the imbalance of Bax/Bcl-2 protein expression. Moreover, NGAL overexpression increased the levels of reactive oxygen species and oxidase activity, but reduced anti-oxidase activity. Mechanistically, we found that NGAL decreased the expression of integrin β3, but not the expression of integrin αvβ3 and αvβ5, thus inhibiting the downstream protein AKT. When we used the constitutively activated AKT overexpression adenovirus to activate AKT, the deteriorated phenotype by NGAL was counteracted.

Conclusions: NGAL can directly affect cardiomyocytes and cause cardiomyocyte deteriorated hypoxia injury through inhibiting integrin β3 signaling.

MeSH Keywords: Cell Hypoxia • Integrin beta3 • Myocytes, Cardiac

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Background

Ischemic heart disease (IHD) is a major cause of mortality and morbidity worldwide [1]. The estimated annual incidence of heart attack caused by myocardial infarction is 865 000 new and recurrent attacks, and the prevalence and mortality due to MI increases with age [2,3]. The prognosis of acute myocardial infarction (AMI) and ischemic cardiomyopathy (ICM) remains poor despite significant advances in medical therapy and revascularization strategies [4]. The need for improved intervention for these patients and for those with acute cardiac ischemia led to the development of molecular-based therapy.

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2 protein, is a 25-kDa protein involved in the host defense against some gram-negative bacteria through binding with iron-loaded bacterial siderophores, which limits the availability of this essential nutrient to bacteria, leading to inhibition of their growth and pathogenicity [5]. NGAL levels have been used to reflect tissue damage, particularly in the kidney, and more recently for cardiovascular disease manifestations, including hypertensive cardiac hypertrophy [6], coronary artery disease [7], and acute heart failure [8]. Oklonomou et al. reported a positive correlation between NGAL and myocardial fibrosis and remodeling in heart failure patients [8]. Shalenkova et al. found that NGAL may be used as a marker for severity of cardiovascular conditions and heart remodeling in patients after exacerbation of ischemic heart disease [9]. NGAL was also reported to participate in the pathology of cardiac hypertrophy and heart failure [10]. These data indicate the potential role of NGAL in cardiovascular disease, but the direct role of NGAL in cardiac ischemia injury is unclear. In this study, we used a cardiomyocyte hypoxia model to elucidate the effect of NGAL in cardiac ischemia injury.

Material and Methods

Neonatal rat cardiomyocytes (NRCMs) culture

NRCMs isolation was performed as described in a previous study [11]. Briefly, hearts from 1- to 2-day-old Sprague-Dawley rats were quickly removed and incubated with 0.125% trypsin–EDTA (Gibco). After digestion 4 times, cells were gathered in DMEM-F12 (Gibco) supplemented with 10% FBS. After 1–2 h of culturing, non-cardiac myocytes were adhered to the plastic. NRCMs were then isolated and moved to new plates and cultured with 1% bromodeoxyuridine for 48 h. To overexpress NGAL, cells were transfected with Ad-NGAL (Vigene Biosciences, Shangdong, China). Then, cells were exposed to hypoxia for 24 h. The hypoxia model was induced as described previously [12]. Cell were treated with constitutively-activated AKT adenovirus (Ad-ca.AKT) (Vigene Biosciences, Shangdong, China) to overexpress activated AKT.

qRT-PCR test

RNA was extracted as previously described [13] using TRIzol™ (Roche Diagnostics, Mannheim, Germany). Briefly, after RNA was isolated and purified, 2 µg of each RNA sample was converted to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). Then, samples were converted to PCR products by using the LightCycler 480 SYBR® Green 1 Master Mix (Roche Diagnostics). The GAPDH gene was used as reference. We used the following primers (Sangon Biotech, Shanghai, China): Primer: TNFα: forward: AGCATGATCCGAGATGTGGAA, reverse: TACACAGAAGGCGGTGTTGGC;
IL-1: forward: GGGTATGAGCGACCTGCTAG, reverse: ACCACTTGTGGCCTATGCTCTG;
IL-6: forward: GTGCTTCTTTGGGAGACTGATG, reverse: ATATCGGTCTGTGTTGGTGTG.
All sequences are listed as 5’- 3’.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to test the release of TNFα, IL-1, and IL-6 (BioLegend) according to the method described in a previous study [11]. An ELISA plate reader (Synergy HT, BioTek, VT) was used to detect the optical density at 450 nm.

Oxidative stress

The level of reactive oxygen species (ROS) generation was measured as previously described (Gu et al., 2017). In brief, 2’, 7’-dichlorofluorescin diacetate (DCFH-DA) was used to map cells. DCFH florescence was detected by an ELISA plate reader. Commercial kits (Beiyotime, Beijing, China) were used to detect total superoxide dismutase (SOD) activity, as well as glutathione peroxidase (Gpx) and malondialdehyde (MDA) levels.

TUNEL staining

TUNEL staining was performed as previously described [12]. Briefly, after cells were fixed and permeabilized, a TUNEL reaction mixture was used to label apoptotic cells. We used 4’, 6 diamidino-2-phenylindole (DAPI) to label nuclei. The numbers of TUNEL-positive cells were calculated in a blinded manner.

Western blot analysis

Western blotting was performed as previously described [12]. Briefly, after cells were lysed, proteins were isolated and purified. A 10% sodium dodecyl sulfate – polyacrylamide page was used for electrophoresis and to isolate protein. Primary antibodies were combined at a dilution of 1: 1000: NGAL; Bax; Bcl-2; cytochrome C and GAPDH, integrin avβ3, avβ5, integrin b3, and P- and T- AKT (All purchased from Cell Signaling Technology (Danvers, MA). The secondary antibodies (1: 10 000 dilution)
used were goat anti-rabbit IRdye® 800 CW IgG and goat anti-mouse IRdye® 800 CW (LI-COR). An infrared Li-Cor scanner was used to scan blots. GAPDH was used as reference.

**Statistical analysis**

SPSS version 19.0 (SPSS Inc., Chicago, IL) was used for data analysis. Data are expressed as mean ± standard error. An unpaired t test was used to assess differences between 2 groups, and one-way ANOVA followed by Tukey’s post-hoc test was used to analyze differences among groups. P<0.05 was considered a significant difference.

**Results**

NGAL increased the expression and release of pro-inflammatory factors in response to hypoxia.

Cardiomyocytes were transfected with NGAL to overexpress NGAL (Figure 1A), and then were exposed to hypoxia for 24 h. We detected the pro-inflammatory cytokines expression level with RT-PCR. A sharp increase in TNFα, IL-1, and IL-6 transcription levels was detected in hypoxia cardiomyocytes. Cells in the Ad-NGAL group showed an increased transcription level of these pro-inflammatory factors (Figure 1B, 1C).

**Figure 1.** NGAL increases the pro-inflammatory factors expression and release in response to hypoxia. (**A**) The protein expression of NGAL in NRCMs transfected with Ad-NGAL (n=6). (**B–D**) Transcription level of pro-inflammatory factors in NRCMs exposed to hypoxia (n=6). (**E–G**) Release of pro-inflammatory factors in NRCMs (n=6). * P<0.05 vs. Normoxia-NC; *# P<0.05 vs. hypoxia-NC.
We then assessed the release of these pro-inflammatory factors in cardiomyocytes exposed to hypoxia. Not surprisingly, hypoxia induced significant release of TNFα, IL-1, and IL-6 in cardiomyocytes, while cells transfected with NGAL revealed more release of TNFα, IL-1, and IL-6 when compared with cells in the NC group (Figure 1D–1G). These results suggest that NGAL acts as a pro-inflammatory factor during hypoxia injury in cardiomyocytes.

**Figure 2.** NGAL accelerates the cardiomyocytes apoptosis in response to hypoxia, (A, B) TUNEL staining and quantitative result in NRCMs exposed to hypoxia (n=5). (C–F) Protein expression level of Bax, Bcl-2, and cytochrome C in NRCMs (n=6).

* P<0.05 vs. Normoxia-NC; * P<0.05 vs. hypoxia-NC.
NGAL accelerated cardiomyocyte apoptosis in response to hypoxia

We next sought to determine whether increased NGAL expression in cardiomyocytes results in an increase in apoptosis phenotype. A significant increase in the apoptosis ratio was observed in cells exposed to hypoxia. In these NGAL-overexpressing cells, we found a significantly higher apoptosis ratio when compared with non-NGAL-overexpressing cells (Figure 2A, 2B). In addition to apoptosis, hypoxia resulted in a significant increase in the expression of the pro-apoptosis protein Bax, and reduced expression of the anti-apoptosis protein Bcl-2, which led to the increased release of cytochrome C (Figure 2C–2F) in hypoxic cells. However, NGAL-overexpressing cells had higher expression of Bax and less expression of Bcl-2, which resulted in more release of cytochrome C when compared with cells in the Ad-NC group that were exposed to hypoxia (Figure 2C–2F). These results indicate that NGAL acts as a pro-apoptosis factor during hypoxia injury in cardiomyocytes.

NGAL increased oxidative stress in response to hypoxia

Oxidative stress is associated with cardiomyocyte hypoxia injury. However, whether NGAL-induced inflammation and
apoptosis is associated with increased oxidative stress is unclear. In the present study, ROS level was detected by DCFH-DA, showing that hypoxia induced a significant increase in ROS level, while NGAL further increased the ROS level in hypoxia cardiomyocytes (Figure 3A, 3B). The anti-oxidant was also detected in cardiomyocytes exposed to hypoxia. SOD and Gpx activity were reduced, while NADPH oxidase activity was increased, in cells exposed to hypoxia. NGAL-overexpressing cells had even lower SOD and Gpx activity and further increased NADPH oxidase activity (Figure 3C–3E).

NGAL affected integrin β3/AKT signaling in response to hypoxia

To elucidate the mechanism underlying the pro-inflammation and pro-apoptosis effects of NGAL, we screened the signaling associated with hypoxia injury. We found that integrin signaling was upregulated during the hypoxia injury in cardiomyocytes, with increased integrin avβ3, avβ5, β3, P-AKT, and T-AKT in NRCMs (n=6). * P<0.05 vs. Normoxia-NC; # P<0.05 vs. hypoxia-NC.

We further explored the downstream molecular of integrin β3, and found that AKT activation was downregulated in hypoxia cells. However, the activated AKT was further decreased in NGAL-overexpressing cardiomyocytes in response to hypoxia (Figure 4A–4E). These data suggest that integrin β3/AKT may partly mediate the effect of BAD phenotype of NGAL on cardiomyocytes hypoxia injury.

AKT activation counteracted the effect of NGAL

Next, we assessed whether activating the survival signaling molecular AKT would save the NGAL-mediated BAD phenotype. Cardiomyocytes were transfected with Ad-ca.AKT to overexpress constitutively activated AKT (Figure 5A). The increased pro-inflammatory cytokines release mediated by NGAL overexpression was completely abolished by Ad-ca.AKT (Figure 5B). Further, NGAL overexpression-induced increased apoptosis was also blocked by Ad-ca.AKT (Figure 5C). The increased oxidative stress induced by NGAL overexpression in hypoxia cardiomyocytes was counteracted by Ad-ca.AKT, as shown by the decreased ROS level and increased SOD and Gpx activity, and

**Figure 4.** NGAL affects integrin β3/AKT signal in response to hypoxia. (A–E) Protein expression of integrin avβ3, avβ5, β3, P-AKT, and T-AKT in NRCMs (n=6). * P<0.05 vs. Normoxia-NC; # P<0.05 vs. hypoxia-NC.
reduced NADPH oxidase activity (Figure 5D, 5E). These data suggest that the NGAL-mediated pro-hypoxia injury effect on cardiomyocytes occurs through integrin β3/AKT signaling.

**Discussion**

This is the first time that a specific cardiomyocytes hypoxia injury effect of NGAL has been demonstrated. Previous studies have focused on the effects of NGAL on coronary artery disease, cardiac hypertrophy, and heart failure [10,14,15]. Nymo reported that serum NGAL concentration is independently associated with mortality in patients with acute coronary syndrome [16]. The level of urine NGAL early after myocardial infarction was also reported to be associated with NT-proBNP concentration in heart failure patients [15]. Moreover, NGAL was recently reported to be the downstream target of a mineralocorticoid receptor that acts as a mediator of post-myocardial infarction cardiac damage [14]. Sung found that NGAL attenuated autophagy to exacerbate cardiac apoptosis induced by myocardial ischemia [7]. However, Zhang reported that NGAL attenuates renal ischemia/reperfusion injury through autophagy.
activation and apoptosis inhibition in rats [17]. These inconsistent results cause confusion about use of NGAL-targeted therapeutic methods in ischemia disease. Our in vitro experiment revealed that NGAL acted as a pro-inflammation, pro-oxidative stress, and pro-apoptosis factor that mediated cardiomyocyte hypoxia injury. These BAD phenotypes caused by NGAL in cardiomyocytes were mediated by regulating integrin β3 signaling.

Integrins are a family of cell-surface receptors composed of non-covalently associated α and β subunits [18]. There are at least 20 different integrin subtypes formed from 12 different α and 8 different β subunits, and many of these subtypes are expressed in a cell type-specific manner, including cardiac myocytes, which express αvβ3, αvβ5, and β3 [19]. A major role of integrins is to physically link the extracellular matrix environment to the intracellular actin cytoskeleton [20]. It is well established that this integrin signaling activates assembly of multiple proteins at the focal adhesion sites regulating cell apoptosis and survival [21]. In our study, we found that during the pathology of hypoxia injury, the expressions of αvβ3, αvβ5, and β3 were increased in cardiomyocytes, while NGAL overexpression suppressed β3 expression but not the αvβ3 or αvβ5 level. This is consistent with the results of Su et al. showing that integrin β3 inhibited hypoxia-induced apoptosis in cardiomyocytes [22]. We then explored the downstream survival signaling of AKT. The AKT pathway is one of the key signaling cascades activated upon insulin-like growth factor receptor (IGF-1R) stimulation [23]. This pathway plays a central role in regulating cell survival [24]. The short cytoplasmic domains in β-integrins serve as docking sites for signaling molecules, including non-receptor tyrosine kinases focal adhesion kinase (FAK), which activates AKT to promote cell survival [25]. In the present study, we observed decreased AKT activation during the hypoxia pathology, and NGAL further reduced AKT activation, thus causing increased cell apoptosis. Moreover, when we used the constitutively activated AKT overexpression adenovirus to transfect cells, the NGAL overexpression that induced all the BAD phenotypes were abolished. This further confirmed that NGAL-induced cell hypoxia injury involves integrin β3/AKT signaling.

Figure 5. AKT activation counteracts the effect of NGAL. (A) Expression level of P-AKT in cardiomyocytes transfected with Ad-ca.AKT (n=6). (B) Release of pro-inflammatory factors in NRCMs (n=6). (C) TUNEL staining and quantitative result in NRCMs (n=5). (D) ROS level detected by DCFH-DA (n=6). (E) SOD, Gpx, and NADPH oxidase activity in NRCMs (n=6). * P<0.05 vs. Normoxia-NC; # P<0.05 vs. hypoxia-NC.
Conclusions

Overall, the present study demonstrates that the increase in NGAL during cardiac ischemia could be a potential mechanism for integrin β3 inhibition, leading to programmed cell death of cardiomyocytes. Therefore, NGAL and its downstream factors involving integrin β3 could be possible targets to prevent cell injury in patients with ischemic heart diseases.

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