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Lymphotoxin- α is a Novel Adiponectin Expression Suppressor Following Myocardial Ischemia/Reperfusion

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Running Title: Regulation of Adiponectin Expression

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Abstract:

Recent clinical observations demonstrate adiponectin (APN), an adipocytokine with potent cardioprotective actions, is significantly reduced following myocardial ischemia/reperfusion (MI/R). However, mechanisms responsible for MI/R-induced hypoadiponectinemia remain incompletely understood. Adult male mice were subjected to 30 minutes MI followed by varying reperfusion periods. Adipocyte APN mRNA and protein expression, and plasma APN and TNF α concentrations were determined. APN expression/production began to decline 3 hours after reperfusion (reaching nadir 12 hours after reperfusion), returning to control levels 7 days after reperfusion. Plasma TNF α levels began to increase 1 hour after reperfusion, peaking 3 hours and returning to control levels 24 hours after reperfusion. TNF α knockout significantly increased plasma APN levels 12 hours after reperfusion, but failed to improve APN expression/production 72 hours after reperfusion. In contrast, TNF receptor-1 (TNFR1) knockout significantly restored APN expression 12 and 72 hours after reperfusion, suggesting other TNFR1 binding cytokines contribute to MI/R-induced APN suppression. Among many cytokines increased after MI/R, Lymphotoxin- α (LT α) was the only cytokine remaining elevated 24-72 hours after reperfusion. LT α knockout did not augment APN levels 12 hours post reperfusion, but did so by 72 hours. Finally, in vitro treatment of adipocytes with TNF α and LT α at concentrations seen in MI/R plasma additively inhibited APN expression/production in TNFR1-dependent fashion. Our study demonstrates for the first time that LT α is a novel suppressor of APN expression and contributes to the sustained hypoadiponectinemia following MI/R. Combining anti-TNF α with anti-LT α strategies may achieve the best effects restoring APN in MI/R patients.

Keywords: Adipokines; Diabetes; Reperfusion Injury; Tumor Necrosis Factor

Early reperfusion after coronary occlusion remains the most effective means of limiting ischemic myocardial injury. However, evidence from animal studies, as well as clinical observations, demonstrates that reperfusion itself may cause additional cell death, defined as “reperfusion injury”(3). Strong epidemiological evidence suggests type-2 diabetes not only causes coronary vascular injury thereby increasing ischemic heart disease prevalence, but also exacerbates cardiac injury after ischemia/reperfusion insult in these patients (2; 8; 15). Clarifying the molecular link between type-2 diabetes and cardiovascular injury may therefore help identify novel effective therapeutic interventions attenuating post-ischemic myocardial injury, reducing myocardial ischemic morbidity, and ultimately decreasing diabetic mortality.

Adiponectin (APN) is a protein hormone primarily produced by adipocytes (18). In contrast to the majority of adipokines (e.g., $\text{TNF}\alpha$), which are significantly increased in diabetic patients and pro-inflammatory, APN is markedly reduced in diabetic patients, and is potently protective of the vasculature (16; 25; 27). Plasma APN levels significantly decrease after tissue injury, such as acute lung injury caused by ovalbumin challenge(26). Numerous epidemiological studies reveal the association between hypoadiponectinemia and increased cardiovascular disease risk in obesity and diabetes(4; 7; 13; 32). Additionally, recent clinical observations demonstrate post-MI plasma APN levels correlate positively with myocardial salvage index and ejection fraction recovery(23). Persistent plasma hypoadiponectinemia post myocardial infarction is predictive of future adverse cardiac events(1). However, the molecular mechanisms responsible for post-MI APN suppression remain unidentified.

$\text{TNF}\alpha$ is a well-recognized pro-oxidative cytokine belonging to the same structural family (C1q/TNF superfamily) as APN. Despite their conformational similarities, these two cytokines exert opposite biological effects, and reciprocally regulate each other’s expression and

production. Clinical observations demonstrate that elevated plasma TNF α is associated with low plasma APN levels(11), and anti-TNF α treatment increases plasma APN(12). Although it is well-known TNF α significantly increases after MI/R, and in vitro experiments demonstrate TNF α inhibits APN mRNA expression(5), whether augmented TNF α post-MI is solely responsible for hypoadiponectinemia has not been previously investigated.

Lymphotoxin- α (LT α , also known as TNF- β) is a member of the TNF family, synthesized primarily by activated T and B lymphocytes(17). Previous studies demonstrate LT α shares the same membrane receptors as TNF α , exerting biological effect largely via TNFR1 and TNFR2 activation(17). However, the effect of LT α on APN expression has never been previously investigated.

Therefore, the aim of the present study was to determine the mechanisms responsible for post-MI hypoadiponectinemia, and to assess the role of TNF α and other cytokines in the regulation of APN after MI.

Materials and Methods

Animals: Male adult wild type (WT, C57BL/6), TNF α knockout (TNF $\alpha^{-/-}$), TNF receptor 1 knockout (TNFR1 $^{-/-}$), and lymphotoxin- α knockout (LT $\alpha^{-/-}$) mice (Body Weight: 25-28 g) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were confirmed by specific primer genotyping. All experiments were performed in adherence with the National Institutes of Health *Guidelines on the Use of Laboratory Animals* and were approved by the Thomas Jefferson University Committee on Animal Care.

Experimental Protocols: Mice were anesthetized with 2% isoflurane, and myocardial ischemia reperfusion (MI/R) was produced by temporarily exteriorizing the heart via a left thoracic incision and placing a 6-0 silk suture slipknot around the left anterior descending coronary artery(28). Slipknot release occurred after 30 minutes MI, and myocardial reperfusion commenced for 1 hour to 7 days. Sham-operated control mice (sham MI) underwent the same surgical procedures, except that the suture placed under the left coronary artery was not tied. At the specified time point after reperfusion, 6 animals in each group were sacrificed. Blood was drawn for ELISA determination of APN, TNF- α , and LT α . The epididymal fat pad was utilized for determination of adipose tissue APN mRNA expression.

Determination of Plasma APN, TNF- α , and LT α Concentrations: Plasma APN level was determined via commercial ELISA kit (ALPCO Diagnostics, Salem, NH) per manufacturer's instructions. Plasma TNF- α and LT α concentrations were determined via mouse TNF- α and LT α ELISA kits (BioLegend, San Diego, CA) per manufacturer's instructions.

Quantitative PCR Analysis of APN Expression in Mouse Adipose Tissues: Total RNA was isolated from epididymal adipose tissue via RNeasy Lipid Tissue Midi Kit (Qiagen, Valencia, CA, USA). Standard PCR protocol was performed utilizing an Applied Biosystems Prism 7900

133 Sequence Detection System. cDNA from WT mice epididymal fat pad were synthesized from 2
134 μg of total RNA and 200 ng of Oilgo dT primer via Superscript III RNase H-Reverse
135 Transcriptase protocol (Invitrogen, Carlsbad, CA, USA). For quantitative PCR, samples were
136 analyzed in triplicate in 15 μl reaction volumes (10 ng of cDNA, 450 nmol of primer, 7.5 μl of
137 Master Mix and water) per standard protocol provided by SyBR® Green PCR Master Mix
138 (Applied Biosystems).

139 Adipocyte Cell Culture: 3T3-L1 pre-adipocytes were grown to confluence in DMEM containing
140 10% FBS and penicillin-streptomycin (100 U/mL) at 37 °C, in a humidified 5% CO₂ atmosphere.
141 Cells were induced to adipocyte differentiation 24 hours after confluence by changing the
142 medium to DMEM supplemented with 10% FBS, 5 $\mu\text{g}/\text{mL}$ insulin, 0.5 mM 3-isobutyl-methyl-
143 xanthine, and 0.25 mM dexamethasone sodium phosphate for 48 hours. Thereafter, cells were
144 grown in medium containing only insulin, and refreshed subsequently every 48 hours. Cells were
145 treated with TNF α and LT α (100 pg/ml) alone, or in combination 9-10 days after differentiation
146 (defined as >90% adipocyte phenotype). The effect of TNF α or LT α upon APN mRNA
147 expression (PCR), protein expression (Western), and secretion (APN concentration in culture
148 medium) was determined 3 to 72 hours after treatment. To knockdown TNFR1 protein
149 expression, 50 nM of TNFR1 siRNA or scramble siRNA (Santa Cruz Biotechnology) were
150 transfected into differentiated 3T3-L1 cells (6 days after differentiation induction) via
151 Lipofectamin 2000 reagent (Invitrogen, Grand Island, NY) per manufacturer's instructions. 72
152 hours after transfection, the efficiency of siRNA-mediated TNFR1 knockdown was determined
153 by Western blot. Cells were then treated with TNF α /LT α as described above.

Western Blot Analysis: Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes, and incubated with primary antibodies (anti-APN and anti-tubulin, Cell Signaling Technology, Danvers, MA, USA) followed by horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA). The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL), and observed with a Kodak Image Station 4000R Pro (Rochester, NY).

Statistical Analysis: All values in the text and figures are presented as means±SEM of *n* independent experiments. Data (except Western blot density) were subjected to one way ANOVA followed by Bonferoni correction for *post-hoc* test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn’s post-hoc test. Probabilities of 0.05 or less were considered statistically significant.

Results

MI/R Inhibits APN Expression/Production: APN mRNA expression began to decline as early as 3 hours after reperfusion, reaching nadir 12 hours after reperfusion (72% reduction compared to sham MI/R group). APN mRNA expression slightly increased 24 and 48 hours post-reperfusion, and then modestly declined again 72 hours after reperfusion. After 7 days reperfusion, APN recovered to levels statistically similar to control (Figure 1A). Plasma APN level trend was similar to adipocyte APN mRNA expression, except plasma APN protein nadir occurred 24 hours after reperfusion (Figure 1B).

Different Effect of TNF α and TNFR1 Knockout upon Post-MI/R APN Suppression: It is well-recognized that MI/R stimulates TNF α production. Moreover, previous in vitro studies have demonstrated that TNF α inhibits APN expression. To determine the causative role of TNF α in post MI/R hypoadiponectinemia, we determined the effect of TNF α knockout upon post-MI/R APN expression. Adipocyte TNF α expression was slightly increased before MI/R in TNF α knockout mice (Figure 2A). TNF α knockout almost completely abolished the inhibitory effect of MI/R upon APN expression 12 hours post-MI/R ($P < 0.01$). However, TNF α knockout had no effect upon APN expression 72 hours after MI/R.

TNF α achieves its biological function via activation of two cell membrane receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Our recent in vitro experimental results demonstrate the inhibitory effect of TNF α upon APN expression is mediated by TNFR1. TNFR1 knockout mice were employed to determine whether TNFR1 knockout may similarly affect in vivo APN expression. TNFR1 knockout had similar effect upon APN expression as TNF α knockout, before and 12 hours after MI/R (Figure 2B). Although TNF α knockout failed to restore APN expression 72 hours after reperfusion, TNFR1 knockout markedly reversed the

inhibitory effect of MI/R upon APN expression 72 hours after reperfusion. These results suggest that while TNF α overproduction is largely responsible for the inhibitory effect of MI/R upon APN expression at an early time point (i.e., 12 hours), other cytokines capable of binding with TNFR1 are responsible for the inhibitory effect of MI/R upon APN expression at a late time point (i.e., 72 hours).

To identify novel molecules inhibiting APN expression, plasma concentrations of TNF α , IL-1 β , IL-6, IL-18, TGF- β , and LT α were determined throughout the MI/R period. Among the cytokines investigated, LT α was an attractive candidate for the following three reasons. First, plasma TNF α levels rapidly increase after reperfusion, returned to control levels 24 hours after reperfusion (Figure 3A), suggesting plasma TNF α may not be responsible for late phase (i.e., 72 hours after reperfusion) APN suppression. Second, although multiple cytokines increased after MI/R (data not shown), LT α was the only protein that remained elevated 48 to 72 hours after reperfusion (Figure 3B), a time point during the second phase of APN suppression, not reversed by TNF α knockout. Third, previous studies have demonstrated that LT α shares the same receptors with TNF α . To obtain direct evidence supporting the role of LT α in late phase APN suppression after MI/R, we determined the effect of LT α knockout upon APN expression. LT α knockout had no effect upon APN expression before MI or 12 hours after reperfusion (Figure 3C). However, APN expression levels were significantly increased 72 hours after reperfusion. Taken together, these results suggest LT α may be responsible for late phase APN suppression after MI/R.

Additive Effect of TNF α and LT α upon Adipocyte APN Expression: The aforementioned in vivo experimental results strongly suggest that TNF α and LT α are two cytokines inhibiting APN expression after MI/R. We next performed in vitro experiments to determine whether these two

cytokines may additively inhibit adipocyte APN expression. Consistent with previous reports, TNF α treatment significantly inhibited APN mRNA expression (Figure 4A), protein expression (Figure 4B), and APN secretion (Figure 4C). Treatment of adipocytes with LT α at concentrations observed in plasma after MI/R significantly inhibited APN mRNA expression (Figure 5A), protein expression (Figure 5B), and APN secretion (Figure 5C). Finally, treatment with TNF α and LT α additively inhibited APN expression and production (Figure 6A-C).

TNFR1 Knockout Significantly Attenuated TNF α /LT α Inhibition of APN Expression: In a final attempt to determine the regulatory role of LT α in adipocyte APN expression, we determined the effect of siRNA-mediated TNFR1 expression knockdown upon TNF α /LT α -mediated inhibition of APN expression. siRNA against TNFR1 effectively inhibited adipocyte TNFR1 expression (79 $\pm$ 4.9%). More importantly, TNFR1 downregulation markedly attenuated the inhibitory effect of TNF α /LT α upon adipocyte APN expression (Figure 7A) and production (Figure 7B).

Discussion

Substantial evidence supports reciprocal inhibitory regulation between TNF α and APN(14). Clinical observations demonstrate elevated plasma TNF α is associated with low plasma APN levels(11), and anti-TNF α treatment increases plasma APN(12). In vitro experiments demonstrate that TNF α markedly inhibits APN mRNA expression and secretion in both dose and time-dependent manner(5). TNF- α also decreases rosiglitazone-stimulated APN promoter activity in adipocytes(31). Several mechanisms have been proposed to explain the inhibitory effect of TNF α upon APN expression(14). Firstly, TNF α decreases APN production by suppressing the expression of several factors promoting APN specifically, such as PPAR γ , C/EBP, SREBP, DsbA-L, and retinoid X receptor- α . Secondly, TNF α induces inhibitory transcription factor IGFBP-3, which suppresses APN transcription and induces insulin resistance. Finally, TNF- α inhibits APN expression by activating JNK and nuclear factor κ B systems. Conversely, APN significantly inhibits TNF α expression/production(19). Early studies demonstrate significantly increased TNF α levels in APN knockout mice(30). Recent studies demonstrate APN inhibits TNF- α production via multiple mechanisms, including inhibiting NF κ -B activation, promoting anti-inflammatory cytokine production, and activating the cyclooxygenase-2/prostaglandin E2 pathway(6; 24). Utilizing a TNF α -knockout approach, we provide the first direct in vivo evidence that TNF α overproduction is responsible for hypoadiponectinemia occurring early after reperfusion. Although other pro-inflammatory cytokines (such as IL-6 and IL-18) have been previously reported to inhibit APN expression in cultured adipocytes (14), the current study does not support the major role of such cytokines in post-MI/R hypoadiponectinemia, as TNF α knockout recovered greater than 80% APN expression 12 hours after reperfusion. A likely explanation for the discrepancy between our

current in vivo experimental results and previously reported in vitro results is that plasma IL-6 and IL-18 levels were only slightly elevated and transient after in vivo reperfusion, whereas prolonged high concentrations of IL-6 and IL-18 were utilized in previous culture cell studies(14). This possibility is supported by our data demonstrating the inhibitory effect of MI/R upon APN expression/production is less than previously reported from in vitro cell culture studies by TNF α (91-97% reduction in APN mRNA expression after 1-3 days of TNF α incubation)(5). Most in vitro cell culture studies employ high TNF α concentrations (in the ng/ml range), with nearly complete inhibition of resultant APN expression. In our study, although in vivo MI/R markedly increased plasma TNF α levels compared to control, such TNF α levels in MI/R animals (at the very maximum) remained in the pg/ml range.

LT α , a member of TNF family, is synthesized primarily by activated T and B lymphocytes(17). It is expressed in either a secreted or membrane bound form exhibiting different affinity for various receptors. The secreted soluble homotrimeric form binds to the TNFR1 and TNFR2 receptors with high affinity, whereas the transmembrane heterotrimeric form (one LT α plus two LT β) binds with high specificity to the LT β receptor (LT β R). LT α mediates a large variety of inflammatory, immunostimulatory, and antiviral responses, affects cell death or differentiation, and provides an important communicative link between lymphocytes and stromal cells(17). Several genetic and clinical studies have demonstrated that variations in the gene encoding LT α affecting its expression and biological function contribute to the risk of coronary artery disease, myocardial infarction, aortic aneurysm formation, and cerebral infarction(10; 22). Recent clinical studies demonstrate LT α gene variability is also associated with features of the metabolic syndrome, including increased level of C-reactive protein, hyperinsulinemia, and dyslipidemia (9).

Multiple indirect evidences suggest the interplay between LT α and APN. LT α is a potent pro-inflammatory factor, whereas APN is a well-recognized anti-inflammatory molecule. Whereas APN knockout increases atherosclerosis formation(20), loss of LT α reduces lesion size by 62% (21). Moreover, LT α gene variability is associated with hypoadiponectinemia(9). Finally, a recent study demonstrates that APN inhibits lymphotoxin-induced adhesion molecules expression in human umbilical vein endothelial cells via inhibition of NF- κ B activation(29). Utilizing LT α knockout mice and combining both in vivo and in vitro approaches, we provide the first direct evidence that LT α is a novel APN expression suppressor and contributes to hypoadiponectinemia occurring late after reperfusion. These results also suggest that the previously reported pro-atherosclerotic role of LT α may be mediated by APN inhibition. Experiments obtaining direct evidence supporting this interesting hypothesis are currently in progress.

In summary, our current study provided direct evidence that TNF α overproduction immediately after MI/R inhibits cardioprotective APN expression/production and is responsible for the hypoadiponectinemia during the early phase of reperfusion. In contrast, increased LT α production occurring after 48 hours reperfusion is a novel suppressor of APN expression/production during the late phase of reperfusion. Moreover, TNFR1 knockout blocked the suppressive effect of both TNF α and LT α upon APN. These experimental results suggest that simultaneous inhibition of TNF α and LT α , or targeted inhibition of TNFR1 function, may be novel approaches restoring APN expression/production in MI/R patients.

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Figure Legends

Figure 1. Time course of adipocyte APN mRNA (A) expression and plasma APN concentrations (B) after reperfusion (hours). n=5 for mRNA expression at each time point, and n=12 for plasma APN at each time point. **P<0.01 vs. control (time 0).

Figure 2. Effect of TNF α (A) and TNFR1 (B) knockout upon adipocyte APN expression after reperfusion. n=5 at each time point. **P<0.01 vs. control (time 0).

Figure 3. Time course of plasma TNF α (A) and LT α (B) after reperfusion, and effect of LT α knockout upon adipocyte APN expression after reperfusion (C). n=12 for plasma TNF α /LT α at each time point, and n=5 for mRNA expression at each time point. **P<0.01 vs. control (time 0).

Figure 4. Effect of TNF α upon APN mRNA expression (A), protein expression (B), and secretion (C) in cultured 3T3-L1 cells. n=8-10 independent experiments at each time point. *P<0.05, **P<0.01 vs. control (time 0).

Figure 5. Effect of LT α upon APN mRNA expression (A), protein expression (B), and secretion (C) in cultured 3T3-L1 cells. n=8-10 independent experiments at each time point. *P<0.05, **P<0.01 vs. control (time 0).

Figure 6. Effect of combined TNF α and LT α treatment upon APN mRNA expression (A), protein expression (B), and secretion (C) in cultured 3T3-L1 cells. n=8-10 independent experiments at each time point. *P<0.05, **P<0.01 vs. control (time 0).

433 **Figure 7.** Effect of TNFR1 knockout upon TNF α /LT α suppression of APN mRNA expression
434 (A) and production (B) in cultured 3T3-L1 cells. n=8-10 independent experiments at each time
435 point. *P<0.05, **P<0.01 vs. control (time 0).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7