In Vivo Effects of Insulin and Free Fatty Acids on Matrix Metalloproteinases in Rat Aorta

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Running title: Effects of Insulin and FFA on MMPs

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ABSTRACT

Objective: Obesity is associated with insulin resistance, hyperinsulinemia, elevated plasma FFA and increased risk for atherosclerotic vascular disease (ASVD). A part of this increased risk may be due to enhanced activation of matrix metalloproteinases (MMPs). Here, we have examined the effects of physiologically elevated levels of insulin and FFA on 3 MMPs and their inhibitors (TIMPs) in aortic tissue of male rats during euglycemic-hyperinsulinemic clamping.

Methods and Results: Hyperinsulinemia increased MMP-2 (~ 6-fold), MMP-9 (~ 13-fold) and membrane type 1-MMP (MT1-MMP, ~ 8-fold), (all Western blots), the gelatinolytic activity (zymography) of MMP-2 (2-fold), while not affecting TIMP-1 and TIMP-2. Insulin increased IRS-1 associated PI3 kinase (PI3 K), extracellular signal-regulated kinases 1/2 (ERK 1/2) and c-jun NH₂ terminal kinase (JNK) (by Western blots with phospho specific antibodies). FFA augmented the insulin mediated increases in MMP-2 (from ~ 6 to ~ 11-fold), MMP-9 (from ~ 3 to ~ 23-fold), MT1-MMP (from ~ 8 to ~ 20-fold), MMP-2 gelatinolytic activity (from 2 to 3-fold), increased JNK and p38 mitogen activated protein kinase (p38 MAPK) activities but decreased insulin mediated activation of PI3 K and ERK 1/2. Raising FFA without raising insulin affected neither MMPs nor TIMPs.

Conclusions: FFA augmented insulin stimulation of the MMP/TIMP balance of three proatherogenic MMPs and increased activities of 2 MAPKs (JNK and p38 MAPK) both of which are known to stimulate the production of proinflammatory cytokines. This may, over time, increase degradation of extracellular matrix and together with inflammatory changes promote development of ASVD.

KEY WORDS. aorta, metalloproteinases, fatty acids, insulin
Many obese people are insulin resistant (1,2). Insulin resistance, on the other hand, is one of the most important risk factors for the development and progression of atherosclerotic vascular diseases (ASVD) (3,4). The relationship between insulin resistance and ASVD, however, is complex. On one hand, insulin resistance is associated with several established risk factors for ASVD such as type 2 diabetes, hypertension, atherogenic dyslipidemia and abnormalities of blood coagulation and fibrinolysis (5). On the other hand, these associations cannot completely explain the obesity/insulin resistance related ASVD risk suggesting that there may be other, as yet unidentified ways by which insulin resistance increases this risk (6). Indeed, there are reasons to believe that insulin resistance may increase ASVD by promoting matrix metalloproteinase (MMP) activity. MMPs are enzymes with proteolytic activity against connective tissue proteins such as collagen, proteoglycans and elastin and there is accumulating evidence, suggesting that they play a key role in the development of atherosclerotic lesions (reviewed in ref. 7). For instance, increased MMP activity is associated with development of neointimal arterial lesions and smooth muscle cell migration (8); diseased human coronary arteries contain increased MMP-2 and MMP-9 and a shift in the balance of MMPs and their major inhibitors (TIMPs) towards extracellular matrix degradation, particularly in the vulnerable shoulder region of plaques (9,10); degradation of the endothelial cell basement membrane by MMPs facilitates infiltration through the endothelium of monocyte/macrophages, which facilitates inflammation (7); vascular small muscle cells from aneurisms contain more MMP-2 than normal cells (11) whereas MMP-2 deficient mice do not develop aneurisms (12); and elevated plasma MMP-9 levels are a predictor of cardiovascular mortality in patients with coronary artery disease (13).

In the current study, we have tested the hypothesis, that high plasma levels of insulin and FFA are important links between insulin resistance and MMP activation. This hypothesis is based on the following considerations: insulin and FFA levels are commonly elevated in obesity (1,2). FFA cause insulin resistance (14); they also increase the expression and release of proinflammatory cytokines (15) which are potent activators of MMPs (7,16); thus it follows that FFA may activate MMPs. We have tested this hypothesis by using fat infusion to produce high plasma FFA levels and insulin resistance and have examined in rat aortic tissue the in vivo effects of acutely elevated plasma levels of FFA and insulin on protein abundance and activity of MMP-2, MMP-9, MT-1 MMP and their major inhibitors TIMP-1 and TIMP-2 and on several insulin signaling pathways.

RESEARCH DESIGN AND METHODS

Adult male Sprague-Dawley rats (300 - 350 g) were purchased from Charles River Laboratories (Wilmington, MA). They were housed in an environmentally controlled room with a 12-h light/dark cycle, where they had free access to standard rat diet (60% carbohydrate, 10% fat, and 30% protein) and water. One week before the studies, the animals were anesthetized (with 5% oxygen and 2% isoflurane). A polyvinyl catheter (internal diameter = 0.02 in) was inserted into the right internal jugular vein and extended to the right atrium. Another catheter was advanced through the left carotid artery until its tip reached the aortic arch. The free ends of both catheters were attached to long segments of steel tubing and tunneled subcutaneously to the back of the neck where they were exteriorized and secured to the skin.
with clips. At the end of the procedure, the catheters were flushed with isotonic saline containing heparin (50 units/ml) and Ampicillin (5 mg/ml) and filled with a viscous solution of heparin (500 units/ml) and 50% dextrose to prevent refluxing of blood into the catheter lumen. All studies were performed in accordance with the guidelines for the use and care of laboratory animals of the Temple University Institutional Animal Care and Use Committee.

The rats were allowed 1 week to recover from the effects of surgery. At that time, they were within 3% of their preoperative weight. Euglycemic-hyperinsulinemic clamps were conducted in the morning after a 14-h overnight fast. Throughout the studies, the animals were allowed to move freely in their cages. All substrates were administered into the venous catheter, and blood samples were obtained from the arterial catheters. After the clamps, the rats were killed by an overdose of isoflurane and the aorta freeze clamped, excised, carefully cleaned of adventitia and frozen at -80°C until assayed.

The following studies were performed:

**Euglycemic-hyperinsulinemic clamps with or without lipid/heparin infusions.** These clamps were performed with awake and unrestrained rats as described (15) with some modifications. Insulin (4.8 mU·kg⁻¹·min⁻¹) was infused through the jugular vein catheter from 0 to 240 min. Glucose concentrations were clamped at euglycemic levels by a variable rate infusion of 25% glucose. Blood glucose levels were monitored with an Elite Glucometer (Bayer, Elkhart, IN), and glucose infusion rates (GIRs) were adjusted every 5-10 min as needed.

Liposyn II, a 20% triglyceride emulsion (Abbott Labs, Chicago, IL) was infused at 0.618 ml/h together with heparin (20 units/h). In the euglycemic-hyperinsulinemic clamps without lipid/heparin infusions, glycerol (143 µmol/h) instead of lipid/heparin was infused to match the glycerol content of Liposyn II.

In control experiments, glycerol/saline or lipid/heparin was infused without insulin and glucose was maintained at ~ 5.5 mmol/l. Glycerol was infused to simulate the glycerol content in Liposyn II. During these studies blood samples (~ 200 µl) were obtained from the carotid artery at ~30, 0, 60, 120, 180, 210 and 240 min. Blood was centrifuged immediately and the red cells were reinfused into the animals.

**Analytical procedures.** Insulin was measured in plasma by radioimmunoassay using rat insulin as standard (Millipore, St. Charles, MO). FFA were measured in plasma to which a lipoprotein lipase inhibitor (Paroxon; Sigma-Aldrich, St. Louis, MO) had been added with a kit from Wako Pure Chemicals (Richmond, VA).

Rat TIMP-1 was determined with an ELISA kit (from R & D Systems, Inc., Minneapolis, MN) and rat TIMP-2 with an ELISA kit (from Calbiochem) following instructions provided by the manufacturers. Absorbance at 450 nm was measured with a microplate reader (Labsystems, Franklin, MA).

**Immunoprecipitation.** A rabbit anti-IRS-1 serum (from Upstate Co., Lake Placid, NY) and Protein A-Agarose beads were used to immune precipitate IRS-1 associated PI3 kinase from aortic extracts (100 µg).

**Western blots.** Aortic tissues were extracted and protein content measured using the Bio-Rad protein assay (BioRad, Richmond, CA). Samples were separated on 4%-20% SDS gels by using a Tris-glycine running buffer (0.2 M Tris-base, 0.2 M glycine, pH 6.8 and 0.1% SDS). The separated samples were then transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The nitrocellulose membranes were blocked in 5% dried milk-TBS containing 0.1% Tween 20 for 1 hour, then incubated with primary antibodies, diluted in 5% dried milk-TBS, 0.1% Tween 20. The primary antibodies were: mouse
antibodies from Calbiochem (San Diego, CA) against the 72 kDa latent and the 66 kDa active forms of MMP-2, the 92 kDa latent and the 68 kDa active forms of MMP-9, and the ~60 kDa MT1-MMP; a rabbit anti-serum (from Upstate Co., Lake Placid, NY) which recognizes the N-SH$_2$ region of PI3 K and the regulatory p85 subunit of PI3 K and a rabbit anti-serum which recognizes rat IRS-1; a rabbit antibody from Cell Signaling (Danvers, MA) which recognizes the active, dually phosphorylated (at threonine 202 and tyrosine 204) forms of ERK 1/2 (44 and 42 kDa, respectively) and a rabbit anti-serum which detects ERK 1/2; a rabbit antibody (from Cell Signaling) which detects the human and rat phosphorylated (at threonine 180 and tyrosine 182) forms of p38-α, β and γ MAPK (43 kDa); and a rabbit anti-serum which recognizes total p38 MAPK and a rabbit antibody (from Cell Signaling) which detects endogenous levels of p46 (JNK 1) and p54 (JNK 2 and 3) dually phosphorylated at threonine 183 and tyrosine 185 and a rabbit anti-serum which detects total JNK protein. Membranes were washed in TBS containing 0.1% Tween 20 and incubated with secondary antibodies for 1 h. Bands were visualized by using an enhanced chemiluminescence detection kit from Amersham Life Sciences (Arlington Height, IL).

**Gelatin zymography.** MMP-2 activities were measured by gelatin zymography. Aortic tissue extracts were loaded onto SDS-PAGE gels containing 1 mg/ml of gelatin under non-reducing conditions and were run at 100 V for 45 min together with molecular weight standards (BioRad). Gels were then washed twice in 2.5% Triton X-100 and incubated overnight in zymogram development buffer (Bio-Rad Co., Hercules, CA). Gels were then stained with Coomassie blue R-250 followed by destaining with 55% methanol and 7% acetic acid.

**Statement of Responsibility.** The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**RESULTS**

**Elevating plasma FFA causes acute insulin resistance (Figure 1).** Plasma glucose concentrations were clamped at ~ 5.4 ± 0.1 mmol/l in all four studies. Insulin rose from 80 ± 20 before to means of 1564 ± 30 and 1659 ± 56 pmol/l, respectively, during hyperinsulinemic clamps with and without lipid/heparin and did not change during saline infusions (168 ± 92 vs. 237 ± 35 pmol/l, NS) or during lipid/heparin (69 ± 15 vs. 121 ± 43 pmol/l, NS).

Plasma FFA remained unchanged during saline infusions (623 ± 48 pre saline vs. 524 ± 13 µmol/l post saline), decreased from 736 ± 72 to 175 ± 20 µmol/l (p < 0.01) in response to insulin and rose from 692 ± 56 to 2138 ± 187 µmol/l (p < 0.001) in response to insulin plus lipid/heparin and from 636 ± 34 to 2138 ± 284 µmol/l, (p < 0.001) during lipid/heparin.

GIR, (the rate of glucose infusion needed to maintain euglycemia during insulin infusions and a measure of insulin stimulated glucose uptake), rose from 0 to 203 ± 9 and 208 ± 11 µmol/kg min, respectively, after 1 h in the two hyperinsulinemic clamps. After that, GIR remained elevated in the hyperinsulinemic clamps without lipid/heparin and decreased ~ 30% (to 144 ± 10 µmol/kg min) at the end of the hyperinsulinemic clamps with lipid/heparin infusions, indicating the development of FFA mediated insulin resistance and confirming previous results (13,14). GIR did not change during saline or lipid/heparin infusions.

**FFAs potently augment insulin stimulation of MMPs (Figure 2).** MMPs are synthesized as inactive pro-zymogens and activated by proteolytic conversion (7,16). The bioactive (66 kDa) MMP-2 (presented as MMP-2/β-actin ratio) did not change during the 4 h saline infusions (0.5 ± 0.1 pre-study vs. 0.4 ±
Effects of Insulin and FFA on MMPs

0.1 end of study), nor during lipidd/heparin infusion (0.4 ± 0.02), increased ~ 6-fold to 3.1 ± 0.3 after 4 h of hyperinsulinemia (p < 0.02) and increased further (~ 11-fold) to 5.4 ± 0.8 (p < 0.002) with infusion of insulin and lipid/heparin.

The bioactive (68 kDa) MMP-9 (presented as MMP-9/β-actin ratio) also did not change during saline infusions (0.36 ± 0.04 vs. 0.4 ± 0.04), nor during lipid/heparin (0.4 ± 0.02), increased ~ 13-fold during hyperinsulinemia (to 4.7 ± 0.6, p < 0.001) and increased further (~ 23-fold) during insulin and lipid plus heparin infusion (to 8.2 ± 1.6, p < 0.05).

MT1-MMP, which activates MMP-2 from the pro to the active form (7, 16), similarly did not change during saline (MT1-MMP/β-actin ratio pre vs. post saline, 0.13 ± 0.1 vs. 0.13 ± 0.05), nor during lipid/heparin (0.14 vs. 0.02), but increased ~ 8-fold in response to insulin (to 1.08 ± 0.05, p < 0.001) and increased even more (~ 20-fold) in response to insulin plus lipid/heparin infusion (to 2.59 ± 0.18, p < 0.001).

FFA inhibits insulin stimulation of MMP-2 in epidydimal (Figure 3, lower panel). We also examined the effects of insulin and insulin plus lipid/heparin on MMP-2 abundance in epidydimal peritoneal fat. The bioactive (66 kDa) MMP-2/β-actin ratio did not change in response to saline (MT1-MMP/β-actin ratio pre vs. post saline, 1.36 ± 0.24, NS) nor to insulin plus lipid/heparin (0.13 ± 0.22, NS) but increased in response to insulin (to 1.95 ± 0.18, p < 0.01).

Neither FFA nor insulin affect TIMP1 and TIMP2. We next examined protein abundance of TIMP-1 and TIMP-2, the physiological inhibitors of MMPs. TIMP-1 did not change in response to saline (pre vs. post saline, 130 ± 5 vs. 144 ± 6 pg/ml, NS) nor in response to lipid/heparin (174 ± 64, NS), insulin (200 ± 66 pg/ml, NS) or insulin plus lipid/heparin (180 ± 52 pg/ml, NS). TIMP-2 similarly did not change in response to saline (pre vs. post saline, 32 ± 14 vs. 33 ± 24 pg/ml, NS), lipid/heparin (32 ± 23, NS) to insulin (33 ± 12 pg/ml, NS) or insulin plus lipid/heparin (32 ± 16 pg/ml, NS). Thus, there were no significant changes in either TIMP1 or TIMP2 in response to saline, insulin or insulin plus lipid infusions.

FFA augment insulin stimulation of the gelatinolytic activity of MMP-2 (Figure 3). Effects of hyperinsulinemia (with and without lipids) on the gelatinolytic activity of MMP-2 was determined with gelatin zymography. MMP-2 gelatinolytic activity did not change in response to saline (18.9 ± 3.4 vs. 19.9 ± 7.7 arbitrary units), increased 2-fold in response to hyperinsulinemia (to 35.7 ± 5.4 arbitrary units, p < 0.025) and increased further (3-fold) in response to insulin plus lipid/heparin (to 57.8 ± 7.9 arbitrary units, p < 0.001). Very similar changes were seen with pro-MMP-2.

FFA inhibit insulin stimulated PI3K and, ERK1/2 (Figure 4). To assess signaling pathways possibly involved in the observed FFA induced enhancement of insulin stimulated MMP-2, MMP-9 and MT1-MMP, we examined effects of insulin and insulin plus lipid/heparin on key components of the IRS/PI3K/Akt cascade and 3 major MAP kinase pathways. As seen in Figure 4, both the IRS-1 association with the p85 regulatory unit of PI3K and ERK 1/2 phosphorylation (indicating activation of the enzymes), increased under the influence of hyperinsulinemia but decreased in response to insulin plus lipid/heparin. This indicated that neither of these pathways were involved in the FFA mediated increase in MMP-2, MMP-9 and MT1-MMP.

FFAs increase JNK and p38 MAPK (Figure 5). Insulin activated JNK, i.e., increased phospho JNK while FFA further enhanced insulin activation of JNK. p38 MAP phosphorylation was not affected by insulin but was potently increased by FFA. Thus, either one or both of these pathways could
have been involved in the FFA mediated stimulation of MMPs.

**DISCUSSION**

*Raising plasma insulin and FFA levels increases MMP activity in aortic tissue.* The main findings in this study were that 1) insulin stimulated the bioactive forms of MMP-2 in aortic tissue ~ 6-fold, of MMP-9 ~ 13-fold and of MT1-MMP ~ 8-fold and 2) that FFA further augmented these increases (to ~ 11-fold, ~ 23-fold and ~ 20-fold, respectively) while at the same time inhibiting insulin stimulation of total body glucose uptake, i.e., causing insulin resistance. Neither insulin nor FFA affected the 2 major MMP inhibitors, TIMP-1 and TIMP-2. The increase in the MMP/TIMP ratio indicated increased activity of these proteinases and this was directly demonstrated for MMP-2 by gelatin zymography. These results indicated that physiologically high plasma insulin and FFA levels, 2 abnormalities characteristically seen in obese, insulin resistant patients, strongly activated several MMPs which have been strongly implicated in the development and progression of ASVD (9-13).

Previous reports on the effects of insulin on MMPs have produced discrepant results. On one hand, in vitro studies have shown that hyperinsulinemia increased MMP-9 and/or MMP-2 activity in human monocytes and cultured rat glomerular mesangial cells (17,18). On the other hand, Dandona et al. have shown, in vivo, that 4 h of euglycemic-hyperinsulinemia (from 78 ± 144 pmol/l) lowered plasma MMP-9 by 18% in 10 obese non-diabetic subjects (19). The reason for these differences are not clear but could possibly be due to differences in tissue vs. plasma MMP levels, species differences and to interorgan differences. In support of interorgan differences of the FFA effects on MMPs, we were able to show that FFA augmented insulin stimulation of MMP-2 activity in the aorta while decreasing it in epicdydal adipose tissue (Figure 2).

We are not aware of previous reports on effects of FFA on MMPs except for a microarray screen of muscle biopsies obtained after 48 h of lipid infusion in normal volunteers which showed increased MMP-2, 11 and 28 and TIMP-1 expression (20).

We have used heparin to promote lipolysis of the infused lipids and to prevent hypertriglyceridemia (21). Heparin has been reported to decrease phorbolester stimulated MMP-9 expression while having no effect on MMP-2 or TIMP-1 (22,23). Thus, it is possible that we may have underestimated the effect of FFA on MMP-9.

*FFA inhibit insulin stimulation of PI3 K and ERK ½.* To investigate pathways through which insulin and FFA may have affected MMPs, we examined key terminal targets of several insulin signaling cascades. Previous reports have implicated insulin or IGF-1 signaling through the PI3K/Akt cascade to be responsible for stimulation of MMP activity (18,24). In vascular tissues, this pathway controls the metabolic actions of insulin (including glucose and amino acid uptake, glycogen synthesis and nitric oxide production) (25) and is inhibited by FFA in major insulin target tissues (14). Our results confirmed in rat aorta that insulin stimulated PI3K and that FFA suppressed the insulin stimulated PI3K activity. This makes it highly unlikely that the FFA enhanced MMP activity observed in this study was mediated through this pathway.

Other reports have implicated mitogen activated kinases (MAPKs) in the insulin stimulation of MMPs (24,26). MAPKs transmit effects of extracellular stimuli (growth factors including insulin, stress, bacteria) from the cell membrane to the nucleus and mainly control cell growth and proliferation (27). Examining effects of insulin and FFA on ERK 1/2, the terminal target of the canonical Ras/Raf/MEK/ERK
Effects of Insulin and FFA on MMPs

1/2 cascade, we found that similar to its effect on PI3K, FFA also inhibited insulin stimulation of ERK 1/2.

There are several possible explanations for the simultaneous inhibition of the PI3K and ERK 1/2 pathways by FFA. First, it has recently been shown that the PI3K/Akt and the Ras/Raf/MEK/ERK pathways can be interdependent by exerting regulatory control on each other through transphosphorylation (28). Second, ERK 1/2 can bind to activated p38 MAPK which prevents their activation by upstream MEK kinases and results in decreased ERK activation (29). Whatever the mechanism, the suppression by FFA of insulin stimulation of both PI3K and ERK 1/2 makes it unlikely that either of these two pathways was involved in the FFA stimulation of MMPs.

**FFA activate JNK and p38 MAPK.** We next examined JNK and p38 MAPK, the terminal targets of 2 other MAPK cascades, which can be activated by environmental and oxidative stress factors (27). We found that acute elevations of plasma FFA levels significantly increased insulin stimulated JNK activity. JNK activity has been shown to be elevated in many animal models of obesity, by insulin, by FFA, endoplasmic reticulum stress, inflammatory cytokines, bacterial LPS and has been implicated as a mediator of obesity associated insulin resistance (30-36). Once activated, JNK upregulates expression of inflammatory genes by activating the AP-1 transcription factor complex.

We also found that p38 MAPK was strongly activated by FFA. In vitro, FFA have been shown to activate p38 MAPK in hepatocytes, cardiac myocytes and endothelial cells (37-40). p38 MAPK plays a central role in many inflammatory disorders and p38 MAPK antagonists are currently being tested in clinical trials for treatment of rheumatoid arthritis, Alzheimer’s disease and inflammatory bowel disease (41).

Our findings supported the concept of selective insulin resistance. This concept is important to explain why hyperinsulinemia, the usual consequence of metabolic insulin resistance, is associated with decreased insulin action on glucose uptake, glycogen synthesis and lipolysis in skeletal muscle, liver and adipose tissue, while remaining uninhibited in vascular tissue with respect to its action on vascular smooth muscle growth and endothelial cell matrix production. We have expanded the concept of selective insulin resistance by showing that it is not always characterized by suppressed of PI3 kinase and uninhibited ERK 1/2 activity, as was originally proposed for obesity associated insulin resistance (42,43) but that, at least in the case of FFA induced insulin resistance, some MAP kinases are inhibited together with PI3 kinase while other MAP kinases are stimulated. Thus, our data suggest that selective insulin resistance is more variable than was believed earlier and that exactly which MAP kinases are inhibited and which are stimulated may depend on the cause for the insulin resistance and the specific tissues involved.

The upstream events leading to activation of these kinases are not entirely clear although there are several possibilities. First, an increase in plasma FFA has been shown to result in intramyocellular accumulation of diacylglycerol and activation of several PKC isoforms including PKC β2 in human muscle (44), PKC δ in rat liver (15) and PKCθ in rat skeletal muscle (45) and in activation of inhibitor of IκB-α (IKK) and activation of NFκB in rat liver (15). Gao et al. have recently shown that the FFA mediated activation of JNK and IKK is PKC dependent (33) suggesting PKC activation as an upstream event. Second, some recent evidence suggests that FFA mediated activation of JNK, and perhaps other serine/threonine kinases, may be, at least partly, mediated by the Toll like receptor-4
Effects of Insulin and FFA on MMPs

(TLR-4) (46). TLR-4 which is essential for the development of innate immunity to pathogens and triggers production of inflammatory cytokines (36). Third, several G protein-coupled receptors including GRP 40 and GRP 120 have been shown to bind FFA (47,48). There is as yet, however, no evidence that these receptors are involved in any of the FFA activities mentioned above.

While the precise mechanisms by which FFA activate these serine/threonine kinases remains to elucidated, some of the consequences have become clear. First, activation of PKC, JNK and IKK can cause insulin resistance (15, 30, 44). This occurs mainly through serine phosphorylation of IRS 1/2 which inhibits insulin signaling (49). Second, activation of either IKK, JNK or p38 MAPK can result in activation NFκB and release of proinflammatory cytokines and chemokines including TNF-α, IL-1β, MCP-1 (15,50). These cytokines can also be induced in macrophages by insulin (51). Cytokines, in turn, promote the activation of MMPs (4,15).

In fact, using the same experimental protocol as in the current study, we have shown in a previous study that acutely elevating plasma FFA levels results in a strong and continuous increase in circulating MCP-1 levels in rats (15). Hence, the effects of FFA and insulin on MMPs are likely to be indirect and mediated through cytokines.

**Clinical Relevance.** In this study we have shown that hyperinsulinemia increased the activities of three MMPs several fold and that the combination of hyperinsulinemia and high plasma FFA levels further augmented these increases. All three of these MMPs have been implicated to play important roles in development and progression of ASVD such as heart attacks, strokes and peripheral arterial diseases. We have also shown that hyperinsulinemia activated JNK and that the combination of hyperinsulinemia and elevated FFA levels further augmented the activation of JNK and in addition, strongly activated p38 MAPK. Activation of these pathways are known to result in the synthesis and release of proinflammatory and proatherogenic cytokines. These findings are pertinent because the combination of high plasma insulin and FFA levels is common in obese, insulin resistant patients whether or not they have type 2 diabetes. This group of individuals also has a greatly increased risk for ASVD compared to non-obese and non-insulin resistant subjects. Assuming that our results obtained in rat aortic tissue are applicable to human vascular tissue, we believe that the increased MMP activity and the activation of several MAP kinases shown here, may over time increase the degradation of extracellular matrix and produce inflammatory changes which together may lead to progression of atherosclerotic lesions and contribute to the increased risk for ASVD in obesity.

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Effects of Insulin and FFA on MMPs


FIGURE LEGENDS

**Figure 1.** Plasma glucose, insulin, FFA levels and rates of glucose infusion needed to maintain euglycemia (GIR) in adult Sprague-Dawley rats during saline infusion (saline, n=3), lipid/heparin infusion (n=3), during euglycemic-hyperinsulinemic clamping with lipid/heparin (insulin/lipid/heparin, n=8) or without concurrent infusion of lipid/heparin (insulin, n=9). Shown are means ± SE.

**Figure 2.** Protein abundance (by Western blots) of aortic MMP-2 (n=6, all 4 studies), MMP-9 (n=6, all 4 studies) and MT1-MMP (n=3 for saline, n=6 for the other 3 studies) in aortic tissues and MMP-2 in peritoneal fat (n=3, all studies) of male Sprague-Dawley rats before (pre) and 4 h after saline infusion (post saline), 4 h after euglycemic-hyperinsulinemic clamping with lipid/heparin (post I/L/H) or without concurrent lipid/heparin infusion (post insulin) and post lipid/heparin (n=3, all studies). Shown are means ± SE of MMP/β actin ratios and representative Western blots (72 kDa, latent MMP2; 66 kDa, active MMP-2; 68 kDa, active MMP-9; 60 kDa, active MT1-MMP). Also shown are tissue inhibitor of MMP-1 (TIMP-1) and MMP-2 (TIMP-2) (lower 2 panels both by ELISA) in rat aorta before (pre) and after 4 h of saline infusion (post saline) and after 4 h of euglycemic-hyperinsulinemic clamping with (post L/H/I) or without concurrent infusion of lipid/heparin (post insulin) and post lipid/heparin (post L/H). Shown are means ± SE (n=6 for all 4 all studies).

**Figure 3.** upper panel - Gelatin-zymography of MMP-2 in aortic tissue from male Sprague-Dawley rats. Shown is one representative zymogram and the mean ± SE of 6 zymograms before (pre) and after 4 h of saline infusion (post saline) and after 4 h of euglycemic-hyperinsulinemic clamping with (L/H) and without (post insulin) concurrent infusion of lipid plus heparin. 72 kDa, latent MMP-2; 66 kDa, active MMP-2.

**Figure 4.** upper panel - Representative Western blots of IRS-1 associated p85 kDa regulatory subunit of PI3K determined after immuneprecipitation with an IRS-1 anti-serum in rat aortic tissue. Bar graphs show means ± SE of IRS-1 associated PI3 K (p85) (n=4 for all studies) before (pre) and post 4 h of euglycemic-hyperinsulinemic clamping with (post L/H) and without (post insulin) concurrent lipid plus heparin infusion.

Middle and lower panel – Representative Western blots of phospho-ERK 1/2 and ERK 1/2. Bar graphs show means ± SE (n=4) of phospho-ERK/ERK-1 (p44) (middle panel) and phospho-ERK-2/ERK-2 (p42) (lower panel).

**Figure 5.** upper panel -Western blots of phospho-e-jun NH2 terminal kinase 2/3 (phospho-JNK-2/3) and JNK-2 and JNK-1 in rat aortic tissues. Bar graphs show means ± SE of phospho-JNK-2/3/JNK-2/3 (n=4) before (pre) and post 4 h of euglycemic-hyperinsulinemic clamping with (post L/H) and without (post insulin) lipid plus heparin infusion.

Lower panel - Representative Western blots of phospho-p38 MAPK and p38 MAPK. Bar graphs show means ± SE of phospho-p38 MAPK/p38 MAPK (n=4) before (pre) and post 4 h of euglycemic-hyperinsulinemia clamping with (post L/H) and without (post insulin) concurrent lipid plus heparin infusion.
Effects of Insulin and FFA on MMPs

- Insulin/Lipid/Heparin
- Insulin
- Saline
- Lipid/Heparin

Fig 1
Effects of Insulin and FFA on MMPs

Fig 2
Effects of Insulin and FFA on MMPs

Fig 3
Effects of Insulin and FFA on MMPs

Fig 4
Effects of Insulin and FFA on MMPs

A

Phospho JNK 2/3 (54 kDa)

JNK 2/3

JNK 1

Phospho JNK 2-3/JNK 2-3

P = 0.004

P = 0.02

Phospho-p38 MAPK

B

p38 MAPK

Phospho-p38 MAPK/p38-MAPK

pre

post insulin

post I/L/H

P = 0.002

P = 0.001

Fig 5