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Leptin-induced cardioprotection involves JAK/STAT signaling that may be linked to the mitochondrial permeability transition pore

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Smith CC, Dixon RA, Wynne AM, Theodorou L, Ong SG, Subrayan S, Davidson SM, Hausenloy DJ, Yellon DM. Leptin-induced cardioprotection involves JAK/STAT signaling that may be linked to the mitochondrial permeability transition pore. *Am J Physiol Heart Circ Physiol* 299: H1265–H1270, 2010. First published July 23, 2010; doi:10.1152/ajpheart.00092.2010.—Leptin-induced protection against myocardial ischemia-reperfusion (I/R) injury involves the activation of the reperfusion injury salvage kinase pathway, incorporating phosphatidylinositol 3-kinase-Akt/protein kinase B and p44/42 MAPK, and the inhibition of the mitochondrial permeability transition pore (MPTP). Recently published data indicate that the JAK/STAT signaling pathway, which mediates the metabolic actions of leptin, also plays a pivotal role in cardioprotection. Consequently, in the present study we considered the possibility that JAK/STAT signaling linked to the MPTP may be involved in modulating the cardioprotective actions of leptin. Employing rat in vitro models (Langendorff-perfused hearts and cardiomyocytes) of I/R injury, we investigated the actions of leptin (10 nM), administered at reperfusion, in the presence or absence of the JAK2 inhibitor, AG-490 (5 μM). Leptin reduced infarct size significantly (control, 60.05 ± 7.41% vs. leptin treated, 29.9 ± 3.24%, $P < 0.05$), protection being abolished by AG-490. Time course studies revealed that leptin caused a 171% ($P < 0.001$) increase in STAT3/tyrosine-705 phosphorylation at 2.5 min reperfusion; however, increases were not seen at 5, 10, 15, or 30 min reperfusion. Contrasting with STAT3, Akt/serine-473 phosphorylation was not significantly increased until 15 min into the reperfusion phase (140%, $P < 0.05$). AG-490 blocked the leptin-induced rise in STAT3 phosphorylation seen at 2.5 min reperfusion but did not influence Akt/serine-473 phosphorylation at 15 min. Leptin reduced the MPTP opening ($P < 0.001$), which was blocked by AG-490. This is the first study to yield evidence that JAK/STAT signaling linked to the MPTP plays a role in leptin-induced cardioprotection. Under the experimental conditions employed, STAT3 phosphorylation appears to have occurred earlier during reperfusion than that of Akt. Further research into the interactions between these two signaling pathways in the setting of I/R injury is, however, required.

myocardium; ischemia-reperfusion injury; Janus-activated kinase/signal transducer and activator of transcription signaling

THE ADIPOCYTOKINE LEPTIN has been shown to protect against ischemia-reperfusion (I/R) injury in various tissues including kidney (9), gut (6), and brain (34). Recently, we reported that leptin, administered at reperfusion, also protects the myocardium against I/R-induced damage (24). As described for other cardioprotective treatments (14), leptin-induced protection was found to involve the activation of the reperfusion injury salvage kinase (RISK) pathway, through phosphatidylinositol 3-kinase (PI3K)-cellular Akt/protein kinase B (Akt) and p44/42 mitogen-activated protein kinase, and the suppression of mitochondrial permeability transition pore (MPTP) opening (24), mechanisms regarded as prerequisites for cardioprotection (14).

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway plays a vital role in modulating the expression of stress-responsive genes by transducing signals received at the cell surface to the nucleus (7, 17). It is active in various organs and tissues and has been implicated in cardiac hypertrophy (18, 23) and cardiomyocyte apoptosis (28). More recently, evidence has been presented indicating that, in addition to the PI3K-Akt and p44/42 pathways, the JAK/STAT pathway may also play an important role in the protection against myocardial I/R injury (3–5, 26). It has been suggested that STAT3 activation, in particular, may be instrumental in protecting the myocardium following an ischemic insult, with a cytokine such as cardiotrophin acting as the cardioprotective stimulus (21, 27).

It is recognized that many of the metabolic actions of leptin are mediated via JAK/STAT activation (10). Given that it has been proposed that the JAK/STAT pathway may play a vital role in cardioprotection (3–5, 26), we investigated whether the cardioprotective actions of leptin, when administered at reperfusion, may also involve JAK/STAT signaling. In addition, in the light of a recent report that STAT3 is present in mitochondria and is required for optimal function of the electron transport chain and, hence, mitochondrial respiration (32), we considered the possibility that leptin-induced protection mediated via the JAK/STAT pathway is linked to MPTP function.

MATERIALS AND METHODS

Animals. Male Wistar rats, aged 4 mo, were used in these studies and were obtained from Charles River UK. Animals were treated in accordance with the Animals (Scientific Procedures) act 1986, published by the UK Home Office, and the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All procedures were approved under Project license number PPL 70/6281 ("Protection of the Ischaemic and Reperfused Myocardium").

Langendorff-isolated perfused rat heart model. Langendorff experiments were performed as described previously (31). Male rats were anesthetized with pentobarbital sodium by intraperitoneal injection (50 mg/kg). Heparin was administered concomitantly to prevent thrombus formation (300 IU). Hearts were rapidly excised and placed in ice-cold buffer and then perfused retrogradely via the aorta at a constant pressure of 100 mmHg with modified Krebs-Henseleit buffer pH 7.3–7.5 at 37°C. The temperature was monitored by a probe inserted into an incision made in the pulmonary trunk, and hearts were maintained at 37 ± 0.5°C. Heart rate was monitored via a latex intraventricular balloon inflated so that an end-diastolic pressure of 5–10 mmHg was achieved. Hearts underwent a stabilization period of

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40 min, followed by 35 min of regional ischemia and 120 min of reperfusion. Regional ischemia was achieved by tightening a 3.0 silk suture placed around the left anterior descending coronary artery until a substantial reduction in heart rate and coronary flow was observed. At reperfusion, hearts were perfused with normal buffer or buffer containing leptin (10 nM) with or without the JAK2 inhibitor AG-490 (5 μM) for the first 30 min of reperfusion. At the end of the reperfusion period, the risk zone was established by a reocclusion of the suture and the introduction of 5% Evans blue dye into the aorta. The hearts were immediately frozen at −20°C and subsequently cut into 2-mm slices. The heart slices were then incubated in a 1% triphenyltetrazolium chloride solution to stain viable tissue. The slices were analyzed using a computerized planimetry package (Summa Sketch II, Summa Graphics, Seymour, CT). Infarct size was expressed as a percentage of area at risk.

Western blot analysis. Rat hearts were perfused for 40 min to allow for stabilization and then subjected to 35 min regional ischemia (see *Langendorff-isolated perfused rat heart model*). Hearts were then reperfused for 2.5, 5, 10, 15, or 30 min, at which time the ventricular tissue at risk was excised and snap frozen in liquid nitrogen before being stored at −80°C to await analysis. On analysis, the proteins were extracted by homogenization followed by high-speed centrifugation, and the resultant were supernatants assayed for protein content using a bicinchoninic acid assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was then used to separate the proteins that were then transferred to Hybond enhanced chemiluminescence nitrocellulose membranes. Total and phosphorylated STAT3/tyrosine-705 and Akt/serine-473 were detected using appropriate primary and secondary antibodies (Cell Signaling Technology, Hitchin, UK) and an enhanced chemiluminescence blotting reagent (Amersham Biosciences, Little Chalfont, UK). The nitrocellulose membranes were then exposed to photographic film, which was scanned, and the intensities of the protein bands, expressed as arbitrary units, were determined by computerized densitometry (NIH Image 1.63). The relative amounts of phosphorylated and total proteins were then calculated.

Isolated cardiomyocytes: MPTP. Ventricular cardiomyocytes (∼1.5–3 million cells per heart) were isolated from Wistar rat hearts by perfusion with a buffer solution containing collagenase (280 U/mg), as described previously (16). A model of oxidative stress was then employed in which cells in six-well culture plates were subjected to an hypoxia-reoxygenation protocol, and the MPTP opening was assessed using tetramethylrhodamine methyl ester, a voltage-sensitive dye that accumulates in metabolically active, but not depolarized, mitochondria (30). Thus cardiomyocytes were transferred from cell culture medium 199 to an ischemic buffer containing (in mM) 1 KH_2PO_4, 10 $NaHCO_3$, 1.2 $MgCl_2 \cdot 6H_2O$, 25 Na-HEPES, 74 NaCl, 16 KCl, 1.2 $CaCl_2$, and 20 Na lactate (pH 6.2) and placed in an hypoxic chamber (1% O_2) for 4 h. In parallel, cardiomyocytes were maintained under normoxic conditions to serve as a control. At the end of the hypoxic period, the ischemic buffer was removed and replaced with medium 199 supplemented with a nonquenching concentration (100 nM) of tetramethylrhodamine methyl ester, and the cells were incubated in an incubator (95% O_2-5% CO_2) at 37°C. Some cells were incubated together with leptin (10 nM) in the absence or presence of AG-490 (5 μM). Meanwhile, other cells were treated with cyclosporin A (200 nM) to serve as a positive control. After 15 min of reoxygenation, phase-contrast images of 20–30 rod-shaped cells for each well were taken using a Nikon Eclipse TE200 microscope, and mean intensities were determined using the NIH Image J 1.63 software. All values were normalized against the mean intensity for cardiomyocytes maintained under normoxic conditions.

Statistical analysis. Data are presented as means ± SE. Generally, comparisons between more than two groups were made using factorial, one-way ANOVA and the Fisher protected least significant difference post hoc test. In some cases, however, the Kruskal-Wallis ANOVA method was used followed by the Dunn's multiple comparison test. Where only two groups were compared, the Student's *t*-test was used. Differences were regarded as statistically significant if a value of $P < 0.05$ was obtained.

RESULTS

Infarct data. In hearts treated with leptin (10 nM), a significant reduction in infarct size, compared with vehicle-treated control hearts, was observed (control, 60.05 ± 7.41% vs. leptin-treated, 29.9 ± 3.24%, $P < 0.05$, $n = 4–10$) (Fig. 1). The cardioprotective effects of leptin were found to be completely blocked by the JAK2 inhibitor, AG-490 (Fig. 1). It should be noted that no difference in infarct size was seen between control hearts and control hearts treated with DMSO (0.02% final concentration), which was used to dissolve AG-490 (Fig. 1).

Western blot data. In time course experiments, leptin was found to elicit a 171% increase in STAT3/tyrosine-705 phosphorylation at 2.5 min reperfusion ($P < 0.001$, $n = 5$; see Fig. 2). At the other reperfusion time points examined (i.e., 5, 10, 15, and 30 min reperfusion), however, significant changes in STAT3 phosphorylation were not detected ($n = 5$ to 6; see Fig. 2). In contrast with the observations made for STAT3, maximal leptin-induced Akt/serine-473 phosphorylation did not occur until later in the reperfusion phase. Thus a statistically significant ($P < 0.05$) 140% increase in Akt/serine-473 phosphorylation was observed following 15 min reperfusion, with non-significant increases in Akt phosphorylation being seen at 5 (+88%) and 10 (+111%) min reperfusion (Fig. 3). As observed with the infarct data, the actions of leptin on STAT3 phosphorylation at 2.5 min reperfusion (i.e., maximal phosphorylation) were blocked by AG-490 (leptin vs. leptin + AG-490, $P < 0.002$, $n = 4$ to 5; Fig. 3). AG-490 was also found to inhibit STAT3 phosphorylation, albeit to a lesser extent, when administered alone (leptin vs. AG-490, $P < 0.02$, $n = 4$ to 5; Fig. 4*A*). AG-490 failed to influence the leptin-induced rise in Akt/serine-473 phosphorylation seen at 15 min reperfusion (Fig. 4*B*). Total STAT3 and Akt levels did not differ significantly between the various treatment groups.

MPTP data. As reported previously (8, 24), the treatment of cells with leptin (10 nM) reduced the MPTP opening ($P < 0.001$, $n = 4$), which in the case of the present study was stimulated by hypoxia-reoxygenation (Fig. 5). The inhibitory effect of leptin was completely blocked by AG-490 (5 μM).

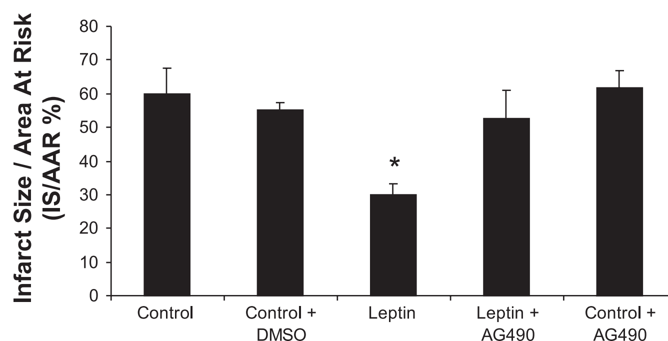

Fig. 1. Abrogation of leptin-induced protection by AG-490. Infarct size is presented as a percentage of the risk zone [%ischemia-reperfusion (%I/R)] in isolated rat hearts perfused with or without leptin (10 nM) during reperfusion (120 min) in the presence or absence of AG-490 (5 μM). The data are presented as means ± SE (*$P < 0.05$ vs. control; $n = 4–10$).

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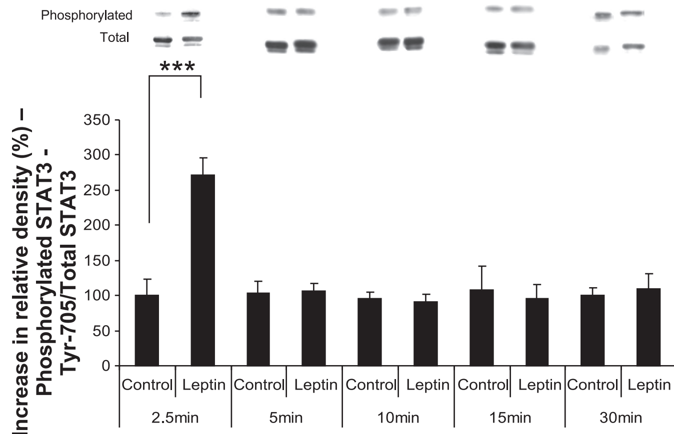

Fig. 2. Phosphorylation of tyrosine-705/STAT3 in the presence and absence of leptin at different time points during reperfusion. Total and phosphorylated STAT3 were determined in extracts derived from rat hearts subjected to I/R under control conditions or in the presence of leptin (10 nM). Data are presented as means ± SE of protein band relative density values normalized to control ($n = 5$ to 6). The values yielded with leptin differed significantly from control when cardiac extracts were run on the same gel (***$P < 0.001$). Representative blots for STAT3 (total and phosphorylated) are included and were chosen from more than one gel. Particular blots were selected because their densitometry values approximated to the mean of the group.

Cyclosporin A (200 nM), an established inhibitor of the MPTP, was used as a positive control in all isolated cardiomyocyte experiments and reduced the MPTP opening to extents comparable with those elicited by leptin ($P < 0.001$, $n = 4$; Fig. 5).

DISCUSSION

We have previously reported that leptin protects the mouse myocardium against I/R injury through a direct action on the heart (24). It has been proposed that the activation of the so-called RISK pathway, which incorporates the PI3K-Akt and p44/42 cascades, is a prerequisite for cardioprotection induced by ischemic preconditioning (IPC) and various pharmacological mediators (14). Indeed, previous studies from this laboratory (8, 24) and the current investigation have yielded evidence that supports this theory further, as the cardioprotective actions of leptin were also found to involve RISK pathway activation (24). Nevertheless, it is generally accepted that cell signaling pathways in addition to PI3K-Akt and p44/42 also underlie cardioprotection. The JAK/STAT pathway, which is known to modulate the metabolic actions of leptin, may constitute one such mechanism (10). In the present study, therefore, we continued our investigations into the cardioprotective actions of leptin but focusing on the JAK/STAT pathway. Additionally, given a recent report that STAT3 present in mitochondria plays a vital role in mitochondrial respiration, we considered

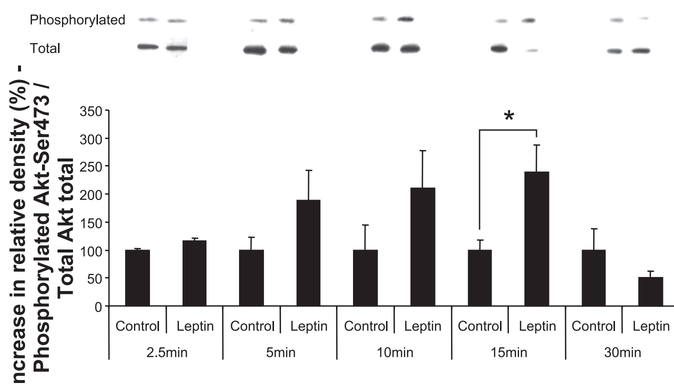

Fig. 3. Serine-473/Akt phosphorylation in the presence and absence of leptin at different time points during reperfusion. Total and phosphorylated Akt were determined in extracts derived from rat hearts subjected to I/R under control conditions or in the presence of leptin (10 nM). Data are presented as means ± SE of relative density values ($n = 5$ to 6). Leptin-treated vs. control values were significantly different when samples were run on the same gel (*$P < 0.05$). Representative blots for Akt (total and phosphorylated) are included and were selected from more than one gel on the basis that their densitometry values were close to the mean.

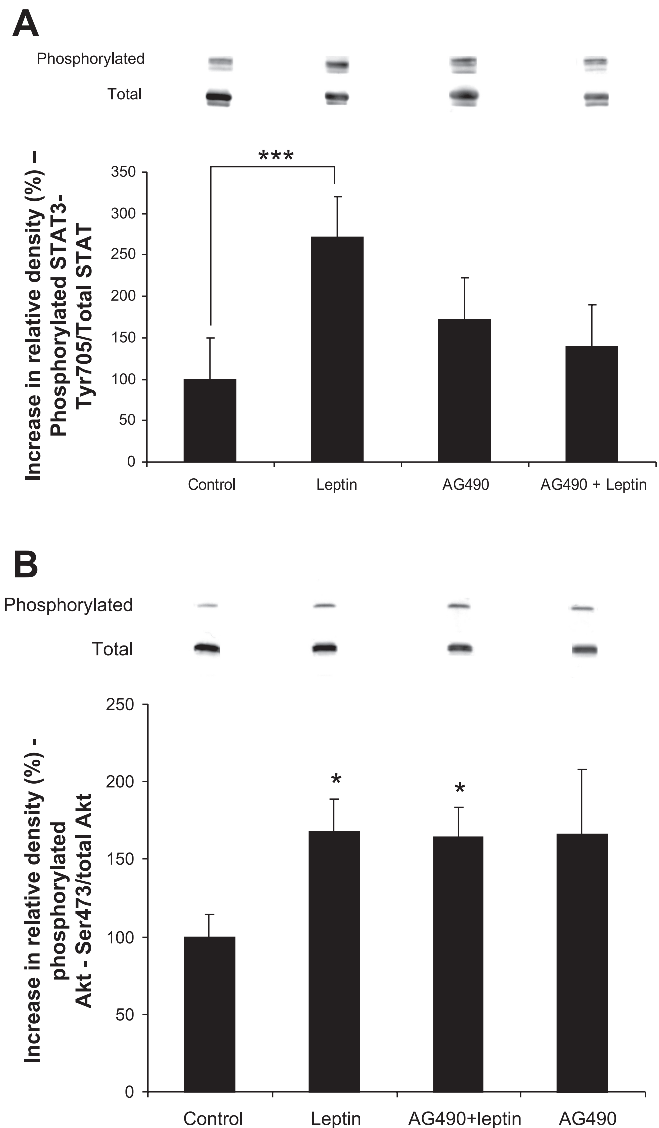

Fig. 4. Influence of the JAK2 inhibitor, AG-490, on leptin-induced phosphorylation of tyrosine-705/STAT3 (*A*) and serine-473/Akt (*B*). The total and phosphorylated forms of STAT3 and Akt were determined in extracts derived from rat hearts subjected to I/R with or without leptin (10 nM) in the presence or absence of AG-490 (5 μM). Data are presented as means ± SE of relative densitometry values in arbitrary units ($n = 4$ to 5). Leptin-treated vs. control values differed significantly when samples were run on the same gel (*$P < 0.05$ and ***$P < 0.002$). Representative Western blots are presented and were selected from more than one gel on the basis that their densitometry readings were close to the mean.

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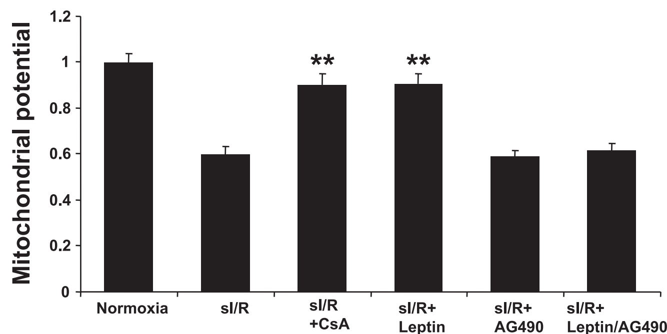

Fig. 5. Leptin-induced inhibition of mitochondrial permeability transition pore (MPTP) opening. MPTP opening in the presence and absence of leptin (10 nM) with or without AG-490 (5 μM) was determined in Wistar rat cardiomyocytes using tetramethylrhodamine methyl ester (100 nM). Cyclosporin A (CsA; 200 nM) was included as a positive control. Data are presented as means ± SE (** $P < 0.001$) and were obtained with a total of 20–30 cells per treatment group from 4 hearts. sI/R, simulated I/R.

the possibility that JAK/STAT signaling and MPTP function are linked. As a consequence, data were obtained indicating that *1*) leptin reduces infarct size via a process involving JAK2 signaling, *2*) leptin-mediated cardioprotection involves STAT3 phosphorylation, and *3*) JAK/STAT signaling is involved in leptin-induced inhibition of the MPTP.

Evidence for JAK/STAT signaling in the heart originally came from studies conducted with rat cardiac myocytes, treatment with leukemia inhibitory factor being found to result in STAT3 activation (18). Subsequently, STAT1 and STAT3 activation was shown to occur in the heart following pressure overload-induced hypertrophy (23). More recently, data consistent with cardioprotection involving the activation of the JAK/STAT pathway were presented. Xuan et al. (33) reported that the late phase of IPC was associated with JAK1 and JAK2 activation followed by STAT1 and STAT3 recruitment and a subsequent upregulation of inducible nitric oxide synthase. These processes were inhibited when the JAK2 inhibitor, AG-490, was administered before IPC. Complementing the study of Xuan and coworkers (33), investigations carried out by Hattori and coworkers (13) demonstrated that the early phase of IPC was associated with JAK/STAT activation accompanied by an upregulation of Bcl-2 and a downregulation of Bax. Once again, AG-490 was found to cause JAK/STAT inhibition (13). Subsequently, it was reported that cardioprotection associated with tumor necrosis factor-α-induced preconditioning, as well as IPC, involves STAT3 activation during the early reperfusion phase (19). Cardiomyocytes genetically depleted of STAT3, while exhibiting degrees of viability similar to wild-type cardiomyocytes, have been shown to be nonresponsive to ischemic and pharmacological preconditioning, as have intact hearts (25). Furthermore, data were recently published (2) showing that cardioprotection stimulated by ischemic postconditioning is lost in STAT-3-deficient and aged mice. Importantly, it was discovered that the blunted responses to the infarct-reducing effects of ischemic postconditioning occurring with aging were accompanied by decreased STAT3 phosphorylation (2). These data, therefore, not only provide evidence that JAK/STAT signaling plays a role in tissue preservation but also indicate a mechanism whereby myocardial damage may develop with age (2).

In the current study, Langendorff experiments performed with Wistar rat hearts revealed that leptin produced significant reductions in infarct size. These data are consistent with previous observations made in the mouse heart and provide additional support for the theory that leptin, administered at reperfusion, might prove of value in the treatment of myocardial infarction (24). The reductions in infarct size induced by leptin were completely blocked when AG-490 was administered at the same time as the adipocytokine, an observation reminiscent of the data of Xuan et al. (33).

Apart from showing that AG-490 blocked the infarct reducing effects of IPC, Xuan and coworkers (33) obtained more direct evidence that the JAK/STAT pathway is involved in cardioprotection. Hence, it was demonstrated that IPC was linked with the rapid tyrosine phosphorylation of JAK1 and JAK2 and the translocation of STAT1 and STAT3 from the cytosol to the nucleus (33). AG-490 blocked both JAK1/JAK2 and STAT1/STAT3 activation, thereby providing even further support for the hypothesis that JAK/STAT signaling plays a substantial role in IPC-induced protection (33). Interestingly, AG-490 treatment was also associated with the inhibition of inducible nitric oxide synthase 24 h after IPC (33). It was therefore concluded that the abrogation of IPC-induced JAK/STAT signaling and subsequent downregulation of inducible nitric oxide synthase might be responsible for the failure by the heart to develop ischemic tolerance. It has become increasingly evident that it is STAT3, rather than STAT1 (which is proapoptotic), which is important with respect to cardioprotection (3). In our study, leptin-stimulated STAT3 phosphorylation appeared to occur in the early stages of the reperfusion phase. This was indicated by our observation that the marked increases in STAT3 phosphorylation seen at 2.5 min reperfusion, following leptin administration, were lost by 5 min. Thus the leptin-stimulated STAT3 signal appeared to be transient, declining rapidly to basal levels. Barry and coworkers (1) have suggested that the increase in JAK/STAT phosphorylation seen in the ischemic heart shortly after the restoration of blood flow is due to a rapid rise in the intracellular levels of reactive oxygen species (as opposed to an upregulation of cytokines or growth factors). It is possible, therefore, that the leptin-induced increase in STAT3 phosphorylation observed in the present study was due to a similar mechanism. As observed by Xuan et al. (33), we found that AG-490 treatment, apart from abrogating the infarct reducing effects of leptin, also inhibited STAT3 phosphorylation.

As already outlined, we have obtained evidence that the PI3K-Akt and p44/42 signaling cascades play important roles in leptin-induced cardioprotection (24). In the present study we obtained further data consistent with this idea, namely, that Akt phosphorylation increased substantially following leptin treatment. We, however, have now demonstrated that the JAK/STAT pathway may also play an important role in leptin-induced cardioprotection. Studies focusing on the interactions between the different cell signaling pathways in the context of myocardial function have been limited. Therefore, one could suggest that detailed investigations into the relationships existing between these cell signaling pathways are needed to gain a greater understanding of the mechanisms underlying cardioprotection. Communication or as it has been termed "cross talk" between cell signaling pathways has been documented in various tissues, including the heart (15, 22). Li et al. (20), for

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example, have shown that cross talk occurs between p44/42 and STAT3 when cardiomyocytes are subjected to cardiotrophin stimulation (20). Suleman et al. (29) demonstrated that a dual activation of STAT3 and Akt is required during the trigger phase of IPC to guarantee cardioprotection. Thus, in preconditioned cardiomyocytes, the inhibition of STAT3 activation with AG-490 was also found to result in Akt inactivation, whereas the inhibition of Akt phosphorylation with wortmannin additionally blocked STAT3 phosphorylation (29). In the current investigation, time course studies indicated that maximal Akt phosphorylation, induced by leptin, occurred later in the reperfusion phase than that of STAT3. One possible interpretation that can be placed on these data is that the JAK/STAT pathway operates upstream of the RISK cascade. Indeed, it has been proposed that JAK/STAT activation, stimulated by postconditioning, may precede Akt phosphorylation and that JAK/STAT phosphorylation in the absence of RISK activation is insufficient to provide protection (12). More recently, cardioprotection induced by insulin was suggested to involve a close interaction between STAT3 and Akt (11). This area of investigation is, however, still in its early stages, and whether JAK-STAT signaling operates upstream of the RISK pathway, in relation to cardioprotection, or the two pathways operate in parallel remains to be established definitively. Certainly, the data obtained in the current investigation appear to be conflicting. Whereas, as outlined above, the Western blot data can be interpreted as being consistent with STAT3 phosphorylation preceding Akt activation, experiments with the JAK2 inhibitor, AG-490, failed to demonstrate that JAK-STAT inhibition was accompanied by Akt blockade. It is important, however, in attempting to reconcile our data with that reported previously to take into account factors such as the types of JAK-STAT inhibitor employed, drug specificity and concentration, and the types of cardioprotective treatments used in the different studies.

Regarding the MPTP data, as reported previously, leptin was found to inhibit pore opening substantially (8, 24). Of more interest, however, was the observation that the JAK2 inhibitor, AG-490, abrogated the effect of leptin on the MPTP opening. These data could be interpreted as indicating that the JAK/STAT signaling pathway and the MPTP are functionally linked. In this regard, one could suggest that the JAK/STAT pathway localized within the cytosol may terminate on the mitochondrion to modulate MPTP function. Alternatively, it is possible that STAT3, which has been reported to be present in mitochondria and is required for the optimal function of the electron transport chain (32), acts to modulate MPTP function in cardiomyocytes.

In conclusion, this is the first study to demonstrate that myocardial protection induced by a pharmacological agent, namely leptin, administered at reperfusion is mediated through a mechanism involving the activation of the JAK/STAT signaling pathway coupled with the inhibition of the MPTP. This finding is, perhaps, unexpected given that the JAK/STAT pathway normally operates at the level of transcription which is, by its nature, a slow process (7, 17). The possibility that alternative cellular mechanisms underlie cardioprotection associated with JAK/STAT activation must, therefore, be considered. Already several potential mechanisms have been proposed, including scenarios in which JAK/STAT activation leads to subsequent activation of the RISK pathway (12, 33). This research is, however, still very much in its initial stages, and detailed investigations into various aspects of cell signaling, including the relative contributions made by these signaling pathways with respect to the cardioprotective mechanisms triggered by leptin and other pharmacological agents, are required to establish which pathways play the predominant roles.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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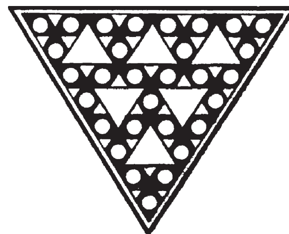

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