Vasopressin is a physiological substrate for the insulin-regulated aminopeptidase IRAP

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The insulin-regulated aminopeptidase (IRAP) is a member of the family of zinc-dependent membrane aminopeptidases and is the homologue of the human placental leucine aminopeptidase (P-LAP) (16). IRAP has a wide tissue distribution (18, 34) but in each of the tissues is expressed only in specific cell types (28). In all cells so far examined, IRAP is found in an intracellular location and is recruited to the cell surface in response to different stimuli (reviewed in Ref. 16). The subcellular localization of IRAP and regulation thereof have been best characterized in adipocytes and muscle cells (reviewed in Ref. 16). In these cells, under basal conditions, IRAP is sequestered within intracellular vesicles that also harbor the insulin-responsive glucose transporter GLUT4. In response to insulin, IRAP, like GLUT4, translocates from the intracellular vesicles to the cell surface. In adipocytes, this results in a 6- to 8- and 10- to 20-fold increase of IRAP and GLUT4, respectively, at the cell surface 5 min after stimulation of cells with insulin.

The physiological significance of the differential subcellular distribution of GLUT4 in basal and insulin-stimulated cells is well established (36). Under fasting conditions, the low number of GLUT4 at the cell surface limits the uptake of glucose into fat and muscle cells, thus making the circulating glucose available as fuel for the brain. After a meal, a maximal number of GLUT4 at the cell surface allows efficient glucose uptake into fat and muscle cells and, consequently, the disposal and storage of 80–90% of the ingested glucose. The differential subcellular distribution of GLUT4 is thus key to the maintenance of glucose homeostasis under fasting and fed conditions.

Little is known about the physiological function of IRAP and the significance of its intracellular sequestration and translocation to the cell surface in response to insulin and other stimuli. Other members of the family of zinc-dependent membrane aminopeptidases, aminopeptidases A and N and the thyrotropin-releasing hormone degrading enzyme, are known to process regulatory peptides in vitro (1, 20, 31, 39). Physiological roles in the cleavage and regulation of the actions of angiotensin II and angiotensin III, respectively, were recently established for aminopeptidases A and N (32). These precedents suggested that a physiological function for IRAP may also be to process one or several regulatory peptides and modify their actions. However, in contrast to the other membrane aminopeptidases that are constitutively expressed at the cell surface (19), the access of IRAP to its extracellular substrates is regulated, by insulin and other stimuli that elicit the translocation of IRAP to the cell surface. In support of this hypothesis, our group and other groups have shown that IRAP and P-LAP cleave several peptide hormones in vitro: vasopressin, oxytocin, lys-bradykinin, angiotensins III and IV, met-enkephalin, and dynorphin A (13, 25, 38). Furthermore, it has been demonstrated that, in insulin-treated adipocytes, in oxytocin-stimulated endothelial cells, and in vasopressin-exposed kidney cells, concomitant with the appearance of IRAP/P-LAP at the cell surface, aminopeptidase activity toward extracellular substrates increases (13, 24, 29).

To establish the physiological function of IRAP, its in vivo substrates needed to be identified. In pursuit of this goal, we compared the cleavage of potential IRAP substrates by IRAP-deficient (IRAP−/−) (17) and wild-type control (IRAP+/+) mice in the absence and presence of insulin. We assumed that

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a peptide that is a physiological substrate for IRAP would not be processed from the NH$_2$ terminus by IRAP$^{-/-}$ mice and cells. Furthermore, we expected that insulin would lead to an increase in the cleavage of an IRAP substrate in IRAP$^{+/+}$ mice and cells but not in IRAP$^{-/-}$ mice and cells.

We tested three peptide hormones that had previously been identified as substrates for IRAP in vitro, vasopressin, oxytocin, and angiotensin IV. These peptides were selected among the potential candidates for the following reasons. Vasopressin and oxytocin, both peptides with an NH$_2$-terminal cysteine that forms a disulfide link with an internal cysteine (Fig. 1), are not cleaved by any of the known aminopeptidases in vitro, except IRAP and P-LAP (19, 20). Since the $K_m$ values of vasopressin and oxytocin for cleavage by purified IRAP and P-LAP are in the low-micromolar range (13), and the two peptide hormones are present only at picomolar concentrations in the circulation (10), it remained questionable whether vasopressin and oxytocin were in vivo substrates for IRAP. Angiotensins III and IV and met-enkephalin are also efficiently cleaved by aminopeptidase N in vitro (6, 39). Although these peptides were not likely specific substrates for IRAP, we included one representative of these, angiotensin IV. The results from our experiments, reported herein, provide strong evidence that vasopressin is a physiological substrate for IRAP.

**EXPERIMENTAL PROCEDURES**

**Materials.** Vasopressin (cat. no. H-1780), oxytocin (cat. no. H-2510) and angiotensin IV (cat. no. H-8125) were obtained from Bachem, and human insulin was from Eli Lilly (HumulinR cat. no. HI-210). (3-[125$I$]-iodotyrosyl$^2$)[Arg$^8$]vasopressin (cat. no. NEX 128), (3-[125$I$]-iodotyrosyl$^2$)oxytocin (cat. no. NEX 187), and (3-[125$I$]-iodotyrosyl$^2$)angiotensin IV (cat. no. NEX 295) were purchased from Perkin Elmer. Protease inhibitors were from Sigma. Organic solvents used were HPLC grade and were obtained from Fisher Scientific. Sep-Pak Vac 3cc (500 mg) C18 cartridges (cat. no. WAT020805) were purchased from Waters. For the vasopressin radioimmunoassay, vasopressin antibodies were obtained from Biogenesis (cat. no. 9536-0225), vasopressin standard was from Peninsula Laboratories (cat. no. 8103), normal rabbit serum was from Roche (cat. no. 100853), and the secondary antibody was provided by Dr. Daniel Haisenleder at the University of Virginia. The details for additional materials are included below.

**Animals.** The generation of IRAP$^{-/-}$ mice is described in detail previously (17). The IRAP$^{-/-}$ and IRAP$^{+/+}$ mice used in the experiments described herein were offspring from heterozygous IRAP$^{-/-}$ breeding pairs or offspring from matched IRAP$^{-/-}$ and IRAP$^{+/+}$ breeding pairs on a mixed BlackSwiss and 129SvEv genetic background. For the experiments described below (see Vasopressin cleavage in vivo), insulin-regulated [125$I$]-inulin in the absence and presence of insulin and Distribution of [125$I$]-inulin in the absence and presence of insulin, mice on a pure 129SvEv background were used. The mice were housed under a constant light (6 AM to 6 PM) and dark cycle, were fed Harlan/Teklad No. 7012 mouse chow, and had free access to water. The animal protocols were approved by the institutional animal care and use committee at the University of Virginia.

**Peptide hormone cleavage by recombinant IRAP.** A recombinant protein consisting of the extracellular portion of IRAP with the catalytic domain (referred to as rIRAP-ED) was produced using the baculovirus-insect cell system as follows. An IRAP cDNA fragment, nucleotides 507–3267 encoding amino acids 146–1026 (18), was subcloned into the BamHI and EcoRI sites in the transfer vector pMelBac (Invitrogen) downstream of and in frame with the honeybee melittin secretion signal sequence to allow the production of a secreted rIRAP-ED protein. The construct was transfected into Sf9 insect cells together with the linearized baculovirus DNA Bac-N-Blue (Invitrogen), according to the manufacturer’s instructions, and a baculovirus containing recombinant viral DNA was obtained. High-Five insect cells growing in serum-free medium in log phase at a density of $7.5 \times 10^4$ to $1 \times 10^5$ cells/cm$^2$ in 75-cm$^2$ flasks were infected with a high-titer stock of the recombinant plaque-puriﬁed baculovirus at a multiplicity of infection of 5. After 24 h of incubation, the medium was removed, the cells were rinsed once with serum-free medium, and fresh serum-free medium was added to the infected HighFive cells. This step was introduced to remove bovine serum albumin (BSA) that was added with the viral stock obtained from Sf9 cells grown in serum-containing medium. After another 48 h of incubation, the medium was collected, and floating cells and cell debris were removed by centrifugation for 5 min at 1,300 g. The cleared medium containing the secreted rIRAP-ED was snap frozen in liquid nitrogen and stored at $-80^\circ$C until further purification. For all of the above-described procedures, Sf9 and HighFive cells were grown as adherent cultures in flasks at 27°C and propagated as described in the manufacturer’s instructions (Invitrogen). The Sf9 cells were grown in serum-containing medium (Grace’s insect me-

![Fig. 1](http://ajpendo.physiology.org/)

Fig. 1. Structures of intact vasopressin (A), oxytocin (B), and angiotensin IV (C) and illustration of their NH$_2$-terminal processing. The sequences of the intact peptides are shown at top; the sequences of the peptides after cleavage of their NH$_2$-terminal peptide bonds are shown at bottom. IRAP, insulin-regulated aminopeptidase; AMPN, aminopeptidase N; *, $^{125}$I-label.
dium, Invitrogen) and the HighFive in serum-free medium (Excell 405, JRH Biosciences).

The rIRAP-ED was purified from the collected medium as follows. On thawing, the medium was concentrated 80-fold with Centricon Plus-80 columns (Millipore). The concentrated medium was applied to a Sephacryl S-300 HR column (HiPrep 16/60, Amersham Biosciences) in aliquots of 2 ml and eluted with 10 mM potassium phosphate, pH 7.5, at a flow rate of 1 ml/min; 2.5-ml fractions were collected. Five-microliter aliquots from each fraction were assayed for aminopeptidase activity toward the fluorogenic substrate L-leucine β-naphthylamide as described previously (17), except that no detergent was added and the aliquots were added directly to 495 μl of buffer containing the substrate at 500 μM. Aminoacidamides were compared with the activity of the native IRAP immunopurified from the medium with a 10% gradient SDS polyacrylamide gel that was stained with Coomassie (Millipore). An aliquot of the concentrated sample was separated on a 10% gradient SDS polyacrylamide gel that was stained with Coo-
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Vasopressin with 33 kDa 125I-labeled vasopressin was injected into the jugular vein in 50 μl of saline, and the tubing was rinsed with 50 μl of saline. Blood samples were taken from the carotid artery at 1, 2, and 3 min after the injection of vasopressin, processed as described above, and subsequently analyzed by TLC.

Analysis of samples by TLC. The medium aliquots and purified plasma samples were analyzed on LC C18 silica gel plates (60 Å, 200-μm thick, Whatman cat. no. 4800-820). Plates were prerun in acetonitrile and dried. The dry plates were placed on a warmplate, and the spots were spotted in the absorbent area of the plates. The plates were developed in 100 mM NaCl and 100 mM triethylammonium acetate (Transgenomics, cat. no. 553303), pH 7.0, in 70% water and 30% acetonitrile. The radioactive signals for the intact peptide hormones and cleavage products were detected by phosphorimaging using the Typhoon 9400 (Molecular Dynamics, Amersham Biosciences). Quantitation of the signals was performed with ImageQuant software (Molecular Dynamics). In some instances, 3-iodo-L-tyrosine (Fluka, cat. no. 58120) was run as a standard on the same plates and visualized by spraying with 0.2% ninhydrin in ethanol (Sigma).

Distribution of [14C]inulin in the absence and presence of insulin. Surgery on mice was performed as described above (see Peptide hormone cleavage in vivo) as were injections (see Vasopressin cleavage in vivo in the absence and presence of insulin), except that the vasopressin was nonradioactive and was injected together with 14C-carboxylated inulin (total 74 kBq/mouse) (Sigma, cat. no. I8259) in 50 μl of saline. Blood samples (100 μl) were collected as described above at 1, 2, 3, 4, and 5 min after the injection of vasopressin and [14C]inulin. Samples were centrifuged for 1 min at 14,000 rpm in a microfuge, and the radioactivity in 40 μl of plasma was determined by liquid scintillation counting. To determine the distribution of [14C]inulin into tissues, mice were euthanized with an intracardiac injection of 100 μl of pentobarbital sodium (50 mg/ml) after the last blood sample had been taken. Kidney, liver, gastrocnemius and soleus muscles, heart, and epididymal or parametrial fat pads were dissected, rinsed in ice-cold phosphate-buffered saline (PBS), blotted dry, frozen in liquid nitrogen, and stored at −80°C. Ten milliliters of scintillation fluid were added to the neutralized solutions, and the samples were counted in a liquid scintillation counter.

Plasma vasopressin levels. IRAP−/− and IRAP+/+ mice had free access to water. Mice were decapitated between 5 and 7 PM, and trunk blood was collected. EDTA was added to the blood to a final concentration of 3 mM, and the plasma was separated by centrifugation for 15 min at 1,600 g in a microfuge at 4°C. The plasma was acidified with hydrochloric acid (final concentration 0.6 N), and insoluble material was pelleted. Peptides were extracted from the plasma with Sep-Pak C18 cartridges according to the manufacturer’s instructions (Waters). In brief, the plasma (maximal 300 μl) was applied to a Sep-Pak C18 cartridge that had been equilibrated in 100% methanol. The loaded columns were extensively washed with PBS, and the peptides bound to the column were eluted in 80% methanol. Samples were dried in a speed vacuum (Eppendorf Vacuum Concentrator 5301) and stored at −80°C until processing. Samples were dried in a speed vacuum (Eppendorf Vacufuge Concentrator 5301) and stored at −80°C until processing. Insoluble material was pelleted by centrifugation for 1 min at maximal speed in a microfuge. Vasopressin was measured in 60- to 100-μl aliquots of the cleared supernatant. Standards with vasopressin concentrations ranging from 0.2 to 100 pg/ml were processed together with the samples, and the amount of vasopressin present in each of the samples was calculated from the standard curve obtained.

Brain vasopressin peptide levels. Brains were dissected from IRAP−/− and IRAP+/+ mice after collection of blood for the measurement of plasma vasopressin levels. The brains were immediately frozen in liquid nitrogen and stored at −80°C until processing, as described previously (15). Briefly, the frozen brains were homogenized with a polytron in ice-cold acid solution (1 M hydrochloric acid, 1% formic acid, 1% trifluoroacetic acid, 1% NaCl) to yield −150 mg tissue/ml homogenate. After insoluble material was pelleted by centrifugation for 15 min at 1,500 g in a microfuge at 4°C, 0.5-ml aliquots of the homogenates were loaded onto Sep-Pak C18 cartridges that had been activated with 100% acetonitrile and washed with 0.2% ammonium acetate (pH 4.0). The loaded columns were washed again with 0.2% ammonium acetate, and the absorbed peptides were eluted in 80% acetonitrile containing 0.1% BSA. Samples were dried and stored at −80°C. The dried samples were resuspended in 0.5 ml of assay buffer, insoluble material was pelleted by centrifugation for 3 min at maximal speed in a microfuge, and 1- to 100-μl aliquots were subjected to the vasopressin RIA as described above (see Plasma vasopressin levels).

Brain vasopressin mRNA levels. Brains were dissected from IRAP−/− and IRAP+/+ mice after withdrawal of blood for the measurement of plasma vasopressin levels. The brains were immediately frozen in liquid nitrogen and stored at −80°C until processing for the preparation of total RNA using Trizol (Invitrogen), following the manufacturer’s instructions. Aliquots of the total RNA were subjected to real-time RT-PCR using vasopressin-specific primers: sense primer (position 20–39 in GenBank sequence gi 6753149), 5′-acctacgctctcgttg-3′, and antisense primer (position 155–172), 5′-cgaaagccgtcttg-3′, located in exons 1 and 2, respectively. One-step RT-PCR was performed using the Quantitect SYBR Green RT-PCR Kit (Qiagen) under the following conditions: RT was performed for 30 min at 50°C, followed by an initial activation step of 15 min at 95°C and 35 PCR cycles with denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Fluorescence data were collected during the extension step. At the end of each run, a melting curve analysis was done. A few representative reactions were separated by agarose gel electrophoresis. Melting curve and agarose gel analysis showed single products with the expected melting temperature of 87°C and size of 153 bp, respectively. To determine the relative vasopressin mRNA expression levels, a standard curve was obtained for one of the IRAP+/+ samples by running reactions with four different amounts of total RNA (12.5, 25, 50, and 100 ng) and plotting the threshold cycles vs. the log of total RNA input. Relative levels of vasopressin mRNA in each sample were derived from the standard curve.

Statistical analysis. Unpaired two-tailed t-tests were used to compare the mean values for the IRAP−/− and IRAP+/+ mice. For the experiment of Vasopressin cleavage in vivo in the absence and presence of insulin (see Fig. 6), the combined data for all time points for IRAP+/+ samples under basal and insulin-stimulated conditions were compared with a two-way ANOVA. Differences were taken to be significant for P values <0.05. Data displayed in the text are expressed as means ± SE.

RESULTS

Peptide hormone cleavage by recombinant IRAP. In all the peptide hormone cleavage studies described herein, we used vasopressin, oxytocin, and angiotensin IV labeled with 125I-tyrosine located in each of the peptide hormones in the second position from the NH2 terminus (Fig. 1). To test whether the labeled peptide hormones were cleaved by IRAP and to establish the products after cleavage by IRAP, the peptide hormones were incubated with purified recombinant IRAP (rIRAP-ED). The reactions were analyzed by TLC, and the radioactive substrates and products were detected by phos-
After 60-min incubation with rIRAP-ED, no intact 125I-vasopressin was detectable (Fig. 2). Eighty-one percent of the total radioactivity was recovered in one major product (designated as VPN in Fig. 2) and 19% in a minor product (designated as Tyr in Fig. 2). The minor product was identified as 3-iodo-[125I]tyrosine by comparing its mobility with the commercially available standard 3-iodo-L-tyrosine. Since IRAP cleaves the peptide bonds in vasopressin sequentially from the NH2-terminus (21, 26), only two radioactive products were expected to be formed in our reaction: vasopressin with the NH2-terminal peptide bond cleaved and the free 3-iodo-[125I]tyrosine (see Fig. 1A). Thus the major product VPN could be identified as 125I-vasopressin with the NH2-terminal peptide bond cleaved. After 60-min incubation of oxytocin with rIRAP-ED, 47% of the total radioactivity was detected as intact 125I-vasopressin, and an equal amount was present in one major product (designated as OTN in Fig. 2). Only 5% of the total radioactivity was recovered as 3-iodo-[125I]tyrosine. Applying the same reasoning as we used for vasopressin, the major product OTN was recognized as 125I-oxytocin with the NH2-terminal peptide bond cleaved (see Fig. 1B). In the reaction of angiotensin IV with rIRAP-ED, no cleavage products were formed, and even after 4 h of incubation, only intact 125I-angiotensin IV was present. In conclusion, these results show that, under identical conditions, rIRAP-ED processes the three peptide hormones differently. rIRAP-ED cleaves the NH2-terminal peptide bond of both vasopressin and oxytocin, more efficiently in vasopressin than in oxytocin. Once the NH2-terminal peptide bonds of vasopressin and oxytocin are cleaved, rIRAP-ED releases the then NH2-terminal 3-iodo-[125I]tyrosine, but with low efficiency. Angiotensin IV is not processed by rIRAP-ED. Our data are in agreement with earlier results obtained after cleavage of unlabeled vasopressin and oxytocin by recombinant and purified IRAP (13, 21, 26). The radiolabeled peptides are thus cleaved by IRAP similarly to their unlabeled counterparts and represent valid tracers to monitor the cleavage of the unlabeled peptides in the reactions. It is possible that the efficiency of cleavage of the radiolabeled peptide hormones is not the same as for the unlabeled peptides. However, this is irrelevant for the studies described below in which peptide hormones are either cleaved or not when tested with IRAP−/− and IRAP+/+ mice and cells under identical conditions.

Peptide hormone cleavage by isolated adipocytes and skeletal muscles in the absence and presence of insulin. Adipocytes and soleus and EDL muscles isolated from IRAP−/− and IRAP+/+ mice were incubated in the absence and presence of insulin in medium containing vasopressin at a concentration of 4 μM and tracer amounts of 125I-vasopressin. Figure 3, A and B, left, shows the analysis by phosphorimaging of media aliquots removed from the adipocytes and soleus muscle, respectively, at the indicated times of incubation. The results for the cleavage of vasopressin by EDL muscles were identical to the results obtained for soleus muscles (data not shown). As can be seen in Fig. 3, A and B, left, in all the samples obtained from the IRAP+/+ adipocytes and soleus muscle, with the exception of the zero time point, the intact radiolabeled vasopressin and two cleavage products can be distinguished (designated as VP, VPC, Tyr). In IRAP−/− adipocyte media, an additional product is detectable (designated as VPN). When comparing the mobilities of these products with the mobilities of the cleavage products obtained with rIRAP-ED (Fig. 2), the two products VPN and Tyr can be identified as 125I-vasopressin with the NH2-terminal peptide bond cleaved and 3-iodo-[125I]tyrosine, respectively. Strikingly, in the samples obtained from IRAP−/− adipocytes and soleus muscles, these two products are not formed, and only the intact radiolabeled vasopressin and one cleavage product (designated as VPC) can be discerned. The relative amounts of the radioactivity detected in 3-iodo-[125I]tyrosine were quantified in samples from three independent experiments, and the results are presented in graphic form in Fig. 3, A and B, right. As explained below, the amount of the 3-iodo-[125I]tyrosine formed is a direct measure for the processing of vasopressin from the NH2-terminus. The relative amounts of the 3-iodo-[125I]tyrosine in the samples from IRAP−/− adipocytes and soleus muscle at all time points, in the presence and absence of insulin, were not significantly higher than the relative background levels detected in the IRAP+/+ samples at time 0. In contrast, the relative amounts of 3-iodo-[125I]tyrosine in the medium of IRAP+/+ adipocytes and soleus muscles increased over the time course of incubation in a linear fashion. In the presence of insulin, the amounts of 3-iodo-[125I]tyrosine produced by IRAP+/+ adipocytes and soleus muscle were 194 ± 41% (n = 3) and 120 ± 4% (n = 3), respectively, of basal values. The quantifications of the relative amounts of intact 125I-vasopressin yielded values that were indirectly proportional to the values obtained for 3-iodo-[125I]tyrosine. For example, in the IRAP+/+ adipocyte samples at the 60-min time point, 61 ± 12 and 33 ± 14% (n = 3) of the total radioactivity were left as intact vasopressin under basal and insulin-stimulated conditions, respectively. At the same time, 33 ± 13 and 54 ± 14% (n = 3) of the total radioactivity were detected in 3-iodo-[125I]tyrosine under basal and insulin-stimulated conditions, respectively. Although equivalent amounts of intact vasopressin and 3-iodo-[125I]tyrosine are detectable, the signal for 3-iodo-[125I]tyrosine appears less impressive. The reason is that it is more spread out than the signal for the intact vasopressin.
our finding that such products are clearly obtained after incubation with IRAP is not dependent on IRAP, and of vasopressin. Since the NH2-terminal cleavage of vasopressin in IRAP is obtained after incubation with IRAP, the NH2-terminal cleavage of vasopressin is dependent exclusively on IRAP, the amount of 3-iodo-[125I]tyrosine produced is a measure for IRAP activity. The formation of the product VPC is not dependent on IRAP, and another peptidase present in adipocytes and skeletal muscles must be responsible for its formation. Burbach and Lebouille (4) demonstrated that, after incubation of vasopressin with brain homogenates and membrane fractions, ~10% of vasopressin was cleaved at the COOH terminus.

Incubation of isolated IRAP+/− and IRAP+/+ adipocytes and soleus and EDL muscles with oxytocin yielded results consistent with the lower efficiency of oxytocin cleavage by rIRAP-ED, relatively less 3-iodo-[125I]tyrosine was produced. Insulin stimulated the cleavage of 125I-oxytocin to a similar extent as the cleavage of 125I-vasopressin.

Figure 4, A and B, show the results for the cleavage of angiotensin IV by IRAP+/+ and IRAP−/− adipocytes and soleus muscles, respectively. Immediately after the addition of angiotensin IV, 3-iodo-[125I]tyrosine was formed in the media obtained from IRAP+/+ adipocytes (time 0). The relative amounts of 3-iodo-[125I]tyrosine, expressed as the percentage of amounts obtained with IRAP+/+ adipocytes minus insulin, were 110 ± 12% for IRAP+/+ adipocytes plus insulin, 111 ± 30% for IRAP−/− adipocytes minus insulin, and 102 ± 4% for IRAP−/− adipocytes plus insulin (n = 3 for each group). Angiotensin IV was also efficiently processed to 3-iodo-[125I]tyrosine by IRAP+/+ and IRAP−/− soleus muscles. The relative amounts of 3-iodo-[125I]tyrosine, expressed as the percentage of amounts obtained with IRAP+/+ soleus muscles minus insulin, were 93 ± 6% for IRAP+/+ soleus muscles plus insulin, 100 ± 2% for IRAP−/− soleus muscles minus insulin, and 111 ± 7% for IRAP−/− soleus muscles plus insulin (n = 3 for each group).
In conclusion, vasopressin and oxytocin are not processed from the NH$_2$ terminus by isolated IRAP$^{-/-}$ adipocytes and skeletal muscles. This suggests that the two peptides are substrates for IRAP expressed in IRAP$^{+/+}$ adipocytes and muscle cells. Consistent with the increased expression of IRAP at the surface of insulin-treated IRAP$^{+/+}$ cells, the cleavages of vasopressin and oxytocin are stimulated by insulin. Angiotensin IV is cleaved with the same efficiency by both IRAP$^{-/-}$ and IRAP$^{+/+}$ adipocytes and muscles and equally well in the absence and presence of insulin. Consequently, the cleavage of angiotensin IV is not dependent on IRAP.

Peptide hormone cleavage in vivo. We next evaluated the clearances of vasopressin, oxytocin, and angiotensin IV from the circulation of the IRAP$^{-/-}$ and IRAP$^{+/+}$ mice. Peptide hormones together with tracer amounts of radiolabeled peptides were injected into the jugular vein of anesthetized random-fed mice, and blood samples were taken from the carotid artery. Figure 5 shows the analysis by TLC and phosphorimaging of samples taken 5 and 20 min after the injections of vasopressin (Fig. 5A) and oxytocin (Fig. 5B) and 1 and 5 min after the injection of angiotensin IV (Fig. 5C). The relative amounts of the $^{125}$I-labeled intact peptide hormones were quantified as a measure for their clearance. At the 5-min time point, 4.1 ± 0.5 ($n = 4$) times more of the intact $^{125}$I-vasopressin was detected in the plasma of IRAP$^{-/-}$ mice than in the plasma of IRAP$^{+/+}$ mice. At the 20-min time point, no intact $^{125}$I-vasopressin was detectable in the IRAP$^{-/-}$ or IRAP$^{+/+}$ plasma. However, a $^{125}$I-labeled vasopressin product was detected at 2.9 ± 1.1 ($n = 4$)-fold higher levels in IRAP$^{-/-}$ plasma compared with IRAP$^{+/+}$ plasma (designated as VPC in Fig. 5A). Comparison of the mobility of the product VPC with the mobilities of the products obtained after cleavage with rIRAP-ED confirmed that it was not vasopressin with the NH$_2$-terminal peptide bond cleaved. At the 5- and 20-min time points, 3-iodo-$^{125}$Ityrosine was readily detectable in IRAP$^{+/+}$ plasma but was absent from IRAP$^{-/-}$ plasma. The results for the clearances of oxytocin and angiotensin IV were clearly different from the data for vasopressin. The same amounts of intact $^{125}$I-oxytocin were detected in IRAP$^{-/-}$ and IRAP$^{+/+}$ plasma at the 5-min time point (116.4 ± 30.0%, $n = 5$). Also, no differences in intact $^{125}$I-oxytocin were observed between IRAP$^{-/-}$ and IRAP$^{+/+}$ mice at earlier time points (1, 2, and 3 min) after the injection of the peptide (data not shown). At the 20-min time point, $^{125}$I-oxytocin was not detectable in both IRAP$^{-/-}$ and IRAP$^{+/+}$ mice. 3-Iodo-$^{125}$Ityrosine was present in the plasma samples of IRAP$^{+/+}$ and IRAP$^{-/-}$ mice at all time points. Angiotensin IV was cleared very efficiently from the circulation of both IRAP$^{-/-}$ and IRAP$^{+/+}$ mice. One minute after injection, no intact $^{125}$I-angiotensin IV was detectable, and only 3-iodo-$^{125}$Ityrosine was present in the plasma of both IRAP$^{-/-}$ and IRAP$^{+/+}$ mice.

In conclusion, the clearance of vasopressin from the circulation of IRAP$^{-/-}$ mice is considerably delayed. The absence of $^{125}$I-vasopressin with the NH$_2$-terminal peptide bond cleaved and 3-iodo-$^{125}$Ityrosine from the IRAP$^{-/-}$ plasma suggests that the delayed clearance is due to compromised NH$_2$-terminal processing of vasopressin in the absence of IRAP. IRAP thus plays a unique role in the cleavage and clearance of vasopressin from the circulation of IRAP$^{+/+}$ mice. VPC is most likely a product obtained by cleavage of the COOH-terminal peptide bonds in vasopressin (4, 8). This product accumulates in the IRAP$^{-/-}$ plasma, like intact vasopressin, because of the lack of processing from the NH$_2$ terminus. In contrast, the clearances of $^{125}$I-labeled oxytocin and angiotensin IV from the circulation of IRAP$^{-/-}$ are the same as in IRAP$^{+/+}$ mice. The presence of 3-iodo-$^{125}$Ityrosine in the plasma samples obtained from male IRAP$^{-/-}$ and IRAP$^{+/+}$ mice (4–6 mo old) 5 and 20 min after injection of vasopressin (A) and oxytocin (B) and 1 and 5 min after the injection of angiotensin IV (C) were analyzed by TLC. In lanes 2 and 3, IRAP$^{+/+}$ samples were loaded. In lanes 5 and 6, IRAP$^{-/-}$ samples were loaded. In lanes 1 and 4, an aliquot of the injected solution (inj) was loaded. Shown for each peptide hormone are the results from 1 representative experiment. The experiments were repeated 4 (vasopressin and oxytocin) and 3 (angiotensin IV) times. Each yielded similar results. In each experiment, the IRAP$^{-/-}$ and IRAP$^{+/+}$ mice used were matched for age and weight. Abbreviations are described in legends to Figs. 2 and 3. The injection of 50 pmol of vasopressin/35 g mouse yielded a pharmacological concentration of ~7 nM in the circulation. However, vasopressin was also cleared efficiently from the circulation of the IRAP$^{+/+}$ mice at a high physiological concentration of 50 pm after injection of a total of 0.4 pmol of vasopressin/35 g mouse. Results (not shown) were identical to data shown in A.
IRAP$^{-/-}$ plasma indicates that these two peptide hormones are processed efficiently in vivo independently of IRAP.

**Vasopressin cleavage in vivo in the absence and presence of insulin.** Considering that the cleavage of vasopressin by isolated adipocytes and skeletal muscles is stimulated by insulin, and the cleavage of circulating vasopressin is mediated by IRAP, we tested the effect of insulin on the cleavage of vasopressin in mice in vivo. Fasted IRAP$^{-/-}$ and IRAP$^{+/+}$ mice were injected with saline or insulin and, 5 min later, with vasopressin. Blood samples were taken at 1, 2, and 3 min after the injection of vasopressin and analyzed by TLC and phosphorimaging (Fig. 6, A and B, left). Figure 6, A and B, right, shows the relative amounts of intact 125I-vasopressin in each sample as a percentage of the intact 125I-vasopressin detected at the 1-min time point in saline-treated IRAP$^{+/+}$ mice. At each time point, significantly less intact 125I-vasopressin was detected in the plasma of insulin-treated than in the plasma of saline-treated IRAP$^{+/+}$ mice: at 1 min, 82 ± 7 vs. 100%; at 2 min, 43 ± 7 vs. 61 ± 6%; and at 3 min, 25 ± 4 vs. 39 ± 3% ($n = 6$). The half-lives of 125I-vasopressin in saline- and insulin-treated IRAP$^{+/+}$ mice were estimated to be ~1.5 and 1 min, respectively. In contrast, 125I-vasopressin was cleared at the same rate from the circulation of saline- and insulin-treated IRAP$^{-/-}$ mice. The estimated half-life for 125I-vasopressin was ~3 min. To test the specificity of the effect of insulin for vasopressin, we also evaluated the clearance of 125I-oxytocin in IRAP$^{+/+}$ mice in the presence and absence of insulin. We found that the clearances of 125I-oxytocin from the circulation of insulin- and saline-treated IRAP$^{+/+}$ mice were the same (data not shown).

In conclusion, insulin increases the clearance of circulating vasopressin by ~30%. Since insulin does not have any effect on the clearance of 125I-vasopressin in the IRAP$^{-/-}$ mice, the insulin-stimulated clearance of 125I-vasopressin in the IRAP$^{+/+}$ mice is most likely the consequence of increased cleavage by IRAP. The effect of insulin is specific for vasopressin; the clearance of oxytocin from the circulation of IRAP$^{+/+}$ mice is not affected by insulin. The three-times-longer half-life for 125I-vasopressin in the fasted IRAP$^{-/-}$ mice corroborates the finding of the delayed clearance of 125I-vasopressin in random-fed mice described above (see **Peptide hormone cleavage in vivo**). The half-life for circulating 125I-vasopressin that we observed in this study is very short. Since 125I-vasopressin binds with lower affinity to vasopressin receptors expressed on the surface of target cells (9), it was possible that the extremely short half-life for the labeled vasopressin was not representative of that for unlabeled vasopressin. However, an earlier study had measured an almost identical short half-life for unlabeled vasopressin (11).

**Distribution of [14C]inulin in the absence and presence of insulin.** The concentration of a peptide in plasma is not only determined by its metabolism but is also a function of its distribution volume and its elimination through excretion. The distribution volume of vasopressin, a small peptide with a molecular weight of 1,000, is most likely determined by the total extracellular fluid space, composed of plasma and interstitial fluid. Vasopressin is predominantly excreted through the kidney (12). To determine whether insulin in combination with vasopressin affected total distribution volume and excretion through the kidney in IRAP$^{+/+}$ mice, we measured the concentration of the extracellular fluid space marker inulin in plasma and kidney after intravenous injection. Inulin has a molecular weight of 5,000, is not modified while circulating in the body, is not taken up into cells, and is excreted relatively slowly through the kidney (7). Fasted IRAP$^{+/+}$ mice were injected with saline or insulin and, 5 min later, with unlabeled vasopressin and [14C]inulin. Blood samples were taken at 1-min intervals for 5 min. Mice were then euthanized and kidneys dissected. Radioactivities in plasma and kidneys were measured. The results, presented in Fig. 7A, show that the [14C]inulin concentrations in IRAP$^{+/+}$ plasma at each time point were the same in the absence and presence of insulin. As shown in Fig. 7B, there was also no significant difference in the amount of [14C]inulin associated with IRAP$^{+/+}$ kidneys from saline- and insulin-treated mice. The amount of radioactivity measured in the kidney was between 15- and 20-fold higher than the amounts of radioactivity found in other major tissues, liver, skeletal muscle, heart, and adipose tissue (data not shown). This finding is consistent with the excretion of inulin.

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**Fig. 6. Vasopressin cleavage in vivo by fasted saline- and insulin-treated IRAP$^{+/+}$ (A) and IRAP$^{-/-}$ (B) mice.** Male and female IRAP$^{+/+}$ and IRAP$^{-/-}$ mice (4–6 mo old) were injected with saline (B) or insulin (1) and, 5 min later, with vasopressin. Plasma samples obtained 1, 2, and 3 min after the injection of vasopressin were analyzed by TLC followed by phosphorimaging. The signals for the intact vasopressin were quantified using ImageQuant, and the values obtained were expressed as percentages of the value obtained for IRAP$^{+/+}$ under basal conditions at 1 min (%IRAP$^{+/+}$ basal). A and B, left: TLC analysis for 1 representative experiment. A and B, right: results for the quantitations (means ± SE) from 6 saline (open bars) and insulin-treated (black bars) IRAP$^{+/+}$ (A) and saline (open bars) and insulin-treated (black bars) IRAP$^{-/-}$ mice (B). For each experiment, mice were matched for sex, age, and weight. Unpaired 2-tailed t-tests were used to compare the mean values for IRAP$^{+/+}$ and IRAP$^{-/-}$ mice at each time point. $p$ values are shown next to the respective bars. The combined data for all time points for IRAP$^{+/+}$ under basal and insulin conditions were compared with a 2-way ANOVA. They were found to be significantly different, with a $p$ value of 0.03. Repetition of the experiments with a lower physiological dose of insulin (2 mU/35 g mouse) yielded identical results.
through the kidney (30). The experiment was repeated with IRAP−/− mice to exclude any contribution of decreased total distribution volumes and decreased excretion to the increased levels of intact vasopressin found in IRAP−/− plasma. The results presented in Fig. 7A show that the [14C]inulin concentrations in IRAP−/− plasma at each time point were the same as in IRAP+/+ plasma. Also, the amounts of radioactivity measured in IRAP−/− kidneys were the same as in IRAP+/+ kidneys (Fig. 7B).

We conclude that insulin does not change the total extracellular fluid space and has no effect on the distribution of inulin into and consequent excretion through the kidney. Thus the decreased concentration of intact 125I-vasopressin in insulin-treated IRAP+/+ plasma is not due to insulin-induced changes in the total distribution volume or excretion and is only due to increased cleavage of 125I-vasopressin. Also, the total distribution volume and excretion in IRAP−/− mice are the same as in IRAP+/+ mice, and, consequently, the increased levels of intact 125I-vasopressin in the IRAP−/− plasma are entirely due to decreased vasopressin cleavage.

**Plasma and brain vasopressin levels.** The results from the experiments described above showed that the clearance of 125I-vasopressin was decreased in the IRAP−/− mice because of impaired processing from the NH2 terminus in the absence of IRAP. To determine how this defect affected endogenous vasopressin, we measured vasopressin levels in the plasma of IRAP−/− mice. To determine how this defect affected endogenous vasopressin, we measured vasopressin levels in the plasma of IRAP+/+ mice. The values were not different whether 2 or 10 mM of insulin or 0.4 or 50 pmol of vasopressin were injected, and the results from the pooled data are shown.

**DISCUSSION**

The present study was undertaken to identify physiological substrates for IRAP. Our experiments demonstrate that the NH2-terminal cleavage of vasopressin by isolated adipocytes and skeletal muscles as well as in the circulation of mice is performed by IRAP, and that the cleavage of endogenous vasopressin by IRAP is crucial in determining circulating vasopressin levels.

**Table 1. Plasma vasopressin and brain vasopressin peptide and mRNA levels in IRAP−/− and IRAP+/+ mice**

<table>
<thead>
<tr>
<th>IRAP−/−</th>
<th>IRAP+/+</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma vasopressin peptide levels, pg/ml</td>
<td>15.6±1.9 (n=8)</td>
<td>7.7±1.0 (n=5)</td>
</tr>
<tr>
<td>Brain vasopressin peptide levels, ng/g tissue</td>
<td>3.75±1.05 (n=12)</td>
<td>12.13±2.89 (n=12)</td>
</tr>
<tr>
<td>Brain vasopressin mRNA levels, %IRAP+/+</td>
<td>50.24±5.4 (n=6)</td>
<td>100±17.15 (n=6)</td>
</tr>
</tbody>
</table>

Data represent means ± SE for the nos. of mice shown in parentheses. Blood samples and brains were obtained from male IRAP−/− and IRAP+/+ mice (5–6 mo old) with free access to water. Peptides were extracted from the plasma and from brain homogenates, and vasopressin concentrations were determined in aliquots of the samples by a radioimmunoassay. Total RNA was extracted from brain and subjected to real-time RT-PCR using primers specific for vasopressin. Mean values for IRAP+/+ and IRAP−/− mice were compared with the unpaired 2-tailed t-test. P values comparing levels between IRAP−/− and IRAP+/+ mice are shown. Mice were matched for age and weight. Mean IRAP+/+ and IRAP−/− brain weights were not significantly different.
It had been recognized early on that vasopressin, after injection into animals, was efficiently eliminated from the circulation (11, 12). The fact that very little intact vasopressin was excreted in the urine indicated that vasopressin was eliminated from the circulation mainly by cleavage (12). Burbach and Lebouille (4), by measuring vasopressin cleavage by brain homogenates and membrane fractions, established that vasopressin was processed by sequential cleavage of amino acids from the NH₂ terminus, typical for aminopeptidase action, and the cleavage of the NH₂-terminal peptide bond of vasopressin was the rate-limiting step in the degradation of vasopressin. An aminopeptidase with vasopressin-degrading activity was subsequently purified from brain tissue and partially characterized (5). However, the identity of this aminopeptidase and the peptidases that were responsible for the observed efficient cleavage of vasopressin in vivo remained unknown (5, 8). Our studies clearly establish IRAP as the aminopeptidase that performs the rate-limiting NH₂-terminal cleavage of vasopressin in vivo and consequently regulates circulating vasopressin levels. Our results on the clearance of vasopressin in the IRAP⁻/⁻ mice also suggest that one or more other peptidolytic pathways exist that lead to the degradation of vasopressin. Although these contribute significantly to the clearance of vasopressin, they are unable to compensate for the absence of cleavage by IRAP in the IRAP⁻/⁻ mice.

Since the cleavage of the NH₂-terminal peptide bond renders vasopressin inactive and unable to bind to vasopressin receptors expressed in peripheral tissues, V₁a, V₁b and V₂ (27, 37), our finding further implies that IRAP also plays a role in the control of vasopressin action. The findings of dramatically decreased levels of vasopressin mRNA and peptide in IRAP⁻/⁻ brain provide evidence for increased vasopressin action. It has been reported that systemically applied vasopressin downregulates its own synthesis in the hypothalamus (33). The decreased vasopressin production in the IRAP⁻/⁻ brains provides an efficient mechanism to maintain plasma vasopressin levels in IRAP⁻/⁻ mice at only twofold increased levels. The necessity and efficiency of the compensatory response to the absence of IRAP suggest a substantial physiological role for IRAP in the regulation of vasopressin levels.

Our data, which show that insulin increases the clearance of vasopressin from the circulation, provide evidence for a role of IRAP in the acute control of circulating vasopressin levels. It is in this effect of insulin that the significance of the subcellular distribution of IRAP becomes evident. As demonstrated by our in vitro cleavage assays, insulin increases the cleavage of vasopressin by isolated adipocytes and skeletal muscles. In an earlier study (13), we had shown that the increase in vasopressin cleavage parallels an increased expression of IRAP at the cell surface of adipocytes. The acute effect on vasopressin cleavage may not be unique to insulin. Other stimuli, including oxytocin in human umbilical vein endothelial cells (29) and vasopressin in kidney cells (24), increase cell surface expression of IRAP and simultaneously augment cleavage of extracellular substrates.

The protection from cleavage in IRAP⁻/⁻ mice in vivo is specific for vasopressin. The processing of oxytocin and angiotensin IV in the circulation is not dependent on IRAP, and either the NH₂-terminal cleavage is not the rate-limiting step in their degradation or it is performed by other aminopeptidases. On the basis of our results with rIRAP-ED and the incubation of these peptide hormones with isolated adipocytes and skeletal muscles, this result was expected for angiotensin IV. The aminopeptidase responsible for the cleavage of angiotensin IV is very likely aminopeptidase N, an aminopeptidase that is ubiquitously expressed (6, 19, 39). However, the result was surprising for oxytocin. Vasopressin and oxytocin have very similar primary and secondary structures; they only differ by two amino acids, positions 3 and 8, and in both, the NH₂-terminal cysteine forms a disulfide bond with an internal cysteine at position 6, forcing the six NH₂-terminal amino acids into a ring structure (Fig. 1). Also, oxytocin, like vasopressin, was cleaved by rIRAP-ED and was not cleaved by adipocytes and skeletal muscle isolated from IRAP⁻/⁻ mice. The only difference that we observed was that oxytocin was processed less efficiently than vasopressin. It is thus possible that the NH₂-terminal cleavage by IRAP is not the rate-limiting step in the degradation of circulating oxytocin. This hypothesis is supported by the finding that oxytocin modified at the NH₂ terminus (deamino-oxytocin), even at high doses, does not have a prolonged effect when injected into rats (35). The action of a similarly modified vasopressin (1-desamino-8-D-arginine vasopressin; dDAVP) is considerably prolonged after injection into rats because of resistance to cleavage from the NH₂ terminus (35).

The human homologue of IRAP, human P-LAP, also known as human oxytocinase, was originally characterized as the enzyme responsible for the cleavage of oxytocin in the plasma of pregnant women. IRAP/P-LAP is well expressed in human placenta and is released into the circulation after cleavage of its extracellular domain from the transmembrane domain (14). Our results do not support a crucial role for IRAP/P-LAP in the cleavage of oxytocin in nonpregnant mice. However, we cannot rule out the possibility that IRAP/P-LAP, when increased in the circulation, still plays a significant role in the degradation of oxytocin during pregnancy.

IRAP has recently been identified as a major binding site for angiotensin IV (2). The physiological significance of this observation is presently not known. Considering that angiotensin IV is rapidly degraded, it is unlikely that angiotensin IV is maintained at high enough concentrations to significantly inhibit the catalytic activity of IRAP in vivo as has been proposed (2). However, it is notable that central application of angiotensin IV and analogs into rats leads to more effective spatial learning (22). Since vasopressin has memory-enhancing effects (3), it is possible that the effects of angiotensin IV and its analogs are mediated by vasopressin. Pharmacological levels of angiotensin IV and its analogs may inhibit IRAP activity sufficiently to cause increases in local vasopressin levels.

In conclusion, we have established vasopressin as a physiological substrate for IRAP. We have uncovered IRAP as the peptidase that controls the efficient clearance of vasopressin from the circulation. Furthermore, our observations suggest that IRAP may be a major player in the control of vasopressin levels and consequently action. We provide evidence that vasopressin levels can be acutely regulated by insulin at the systemic and at the cellular level, most likely through the regulation of the subcellular distribution of IRAP. The physiological significance of the control of vasopressin levels and action by IRAP under basal conditions, as well as the acute modification of vasopressin levels and action by insulin and other stimuli, remains to be determined.
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