Intermedin Restores Hyperhomocysteinemia-induced Macrophage Polarization and Improves Insulin Resistance in Mice*

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Hyperhomocysteinemia (HHcy) is a condition characterized by an abnormally high level of homocysteine, an inflammatory factor. This condition has been suggested to promote insulin resistance. To date, the underlying molecular mechanism remains largely unknown, and identifying novel therapeutic targets for HHcy-induced insulin resistance is of high priority. It is well known that intermedin (IMD), a calcitonin family peptide, exerts potent anti-inflammatory effects. In this study, the effects of IMD on HHcy-induced insulin resistance were investigated. Glucose tolerance and insulin tolerance tests were performed on mice treated with IMD by minipump implantation (318 ng/kg/h for 4 weeks) or adipocyte-specific IMD overexpression mice (Adipo-IMD transgenic mice). The expression of genes and proteins related to M1/M2 macrophages and endoplasmic reticulum stress (ERS) was evaluated in adipose tissues or cells. The expression of IMD was identified to be lower in the plasma and adipose tissues of HHcy mice. In both IMD treatment by minipump implantation and Adipo-IMD transgenic mice, IMD reversed HHcy-induced insulin resistance, as revealed by glucose tolerance and insulin tolerance tests. Further mechanistic study revealed that IMD reversed the Hcy-elevated ratio of M1/M2 macrophages by inhibiting AMP-activated protein kinase activity. Adipo-IMD transgenic mice displayed reduced ERS and lower inflammation in adipose tissues with HHcy. Soluble factors from Hcy-treated macrophages induced adipocyte ERS, which was reversed by IMD treatment. These findings revealed that IMD treatment restores the M1/M2 balance, inhibits chronic inflammation in adipose tissues, and improves systemic insulin sensitivity of HHcy mice.

Intermedin (IMD), also known as adrenomedullin 2 (AM2), is a 53-amino acid peptide belonging to the calcitonin gene-related peptide/calcitonin family (1, 2). IMD shares a common family of G-protein-coupled receptors with another calcitonin gene-related peptide/calcitonin family member, adrenomedullin (AM), which is involved in obesity and its related metabolic disorders (3–5). IMD regulates the central and peripheral circulation and water-electrolyte homeostasis and protects the myocardium from ischemia-reperfusion injury by inhibiting oxidative stress in both the heart and kidney (6–9). The observation of decreased IMD in the plasma of diabetic rats linked IMD with diabetes (8). Previous studies from our group demonstrated that IMD inhibits scavenger receptors and foam cell formation of macrophages and, consequently, protects mice from atherosclerosis (10, 11). Recently published data from our group showed that IMD reduced insulin resistance in high-fat diet-induced obese mice through elevating thermogenesis in brown adipose tissue (12).

Chronic inflammation is considered a pathological issue for aggravating insulin resistance (13, 14). However, the effect of IMD on inflammation aggravated insulin resistance remains largely unknown. In a bacterial LPS-induced atypical orchitis rat model, IMD attenuated the expression of pro-inflammatory cytokines TNFα, IL-6, and IL-1β (15). Down-regulation of IMD was observed in atopic dermatitis (16). These results showed that IMD exerts potential anti-inflammatory effects. In this study, we explored the effects of IMD on macrophage inflammation and adipose insulin resistance.

Hyperhomocysteinemia (HHcy) indicates a plasma homocysteine (Hcy) level >10 μmol/liter. Evidence showed that Hcy induced the production of several pro-inflammatory cytokines in macrophages in both mice and humans (17–20). A meta-analysis and Mendelian randomization analysis from 4011 cases and 4303 controls showed a causal association of Hcy level with the development of type 2 diabetes in humans (21). Our previous studies revealed that HHcy induces insulin resistance and adipokine resistin elevation from adipocytes (22). In a mechanism study of HHcy promoting insulin resistance, endoplasmic reticulum stress (ERS), inflammation pathway activation, and macrophage infiltration were found to be induced by HHcy in adipocytes and adipose tissues (23).

*This work was supported by the National Natural Science Foundation of China Grants 31230035 (to X. W.), 81470554 (to C. J.), and 81401168 (to Y. P.); National Basic Research Program (973 Program) of China Grant 2012CB518002 (to M. X.); and 111 Project of the Chinese Ministry of Education Grant B07001. The authors declare that they have no conflicts of interest with the contents of this article.

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3 The abbreviations used are: IMD, intermedin; HHcy, hyperhomocysteinemia; Hcy, homocysteine; ERS, endoplasmic reticulum stress; PERK, protein kinase R-like endoplasmic reticulum kinase; iNOS, inducible nitric oxide synthase; GTT, glucose tolerance test; ITT, insulin tolerance test; HOMA, homeostasis model assessment; tg, transgenic; eWAT, epididymal white adipose tissue(s); AMPK, AMP-activated protein kinase; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein.
Macrophages play an essential role in regulating the inflammatory state in adipose tissues. Insulin resistance was promoted by a transition in macrophage polarization from an alternative M2 state to a pro-inflammatory M1 state (13, 14, 24, 25). It has been reported that HHcy promotes inflammatory monocyte differentiation (19). Combined with LPS, Hcy induced the M1 polarization of a macrophage cell line, RAW264.7, in vitro (26). However, whether HHcy regulates the M1/M2 balance in insulin-resistant mice and the effects of IMD in HHcy-induced insulin resistance remain unknown.

Our study aimed to explore the mechanisms of HHcy-enhanced ERS and the consequent insulin resistance in adipocytes and the effects of IMD on these processes, revealing IMD as a novel factor on HHcy-induced insulin resistance in adipose tissues of mice.

**Experimental Procedures**

**Materials**—DL-Homocysteine was from Sigma. Antibodies against pAKT (Ser-473), AKT, phosphorylated protein kinase R-like endoplasmic reticulum kinase (pPERK), PERK, p-eIF2α, eIF2α, GPDH, β-actin, p-JNK, p-65, pAMPKα, and AMPKα were from Cell Signaling Technology (Danvers, MA). The antibody against inducible nitric oxide synthase (iNOS) was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ATF6 and arginase 1 (Arg1) were from Abcam (Cambridge, UK). The antibody against t-JNK was from Invitrogen. Recombined human IMD and the antibody against IMD were from Phoenix Pharmaceuticals (Belmont, CA).

**Animal Model**—All studies followed the guidelines of the Animal Care and Use Committee of Peking University and the Guide for the Care and Use of Laboratory Animals. For the HHcy mouse model, male C57BL/6J mice, 6 weeks old, were fed normal mouse chow with or without 1.8 g/liter DL-Hcy added to the drinking water for 4 weeks, as described previously (20, 23). Alzet osmotic minipumps (model 2004) containing 318 ng/kg/h IMD or saline were implanted subcutaneously into male C57BL/6J mice for infusion. After 3 days, the mice were fed Hcy in drinking water for 4 weeks. For adipocyte-specific IMD overexpression, (Adipo-IMD-tg) mice, human intermediary gene expression was driven by the Fabp4 promoter in mice with a C57BL/6j background. For the glucose tolerance tests (ITTs), mice were fasted for 12 h and then injected with glucose (3 g/kg body weight) intraperitoneally. For the insulin tolerance test (ITT), mice were fasted for 4 h, and then 2 IU/kg insulin was injected intraperitoneally. Blood glucose levels were evaluated at 0, 15, 30, 60, 90, or 120 min. After 3 days of adaptation, mice were killed. Blood, epididymal fat pads, and subcutaneous fat pads were taken. Body composition was measured in nonanesthetized mice using an EchoMRITM device (Echo Medical Systems). The homeostasis model assessment (HOMA) index was calculated by using the following formula: [fasting insulin (mIU/l) × fasting glucose (mmol/liter)]/22.5.

**ELISA**—Mouse plasma IMD was detected using an ELISA kit from Cloud-Clone Corp. No significant cross-reactivity or interference between IMD and analogues was observed.

**Quantitative Real-time PCR and Western Blotting Analysis**—Real-time PCR amplification involved the use of an Mx3000 Multiplex quantitative PCR system (Stratagene Corp., La Jolla, CA) and SYBR Green I reagent with normalization to the internal control β-actin. The following primers for real-time PCR were used: Cddl, 5′-GGATCTGGAGTCTGAAATC-3′ (sense) and 5′-CAGTCATGAGTGTGCTA-3′ (antisense); Cddv, 5′-CAGGGTGGGTCCTAGTGTAGT-3′ (sense) and 5′-TGGCATGTCCTGGAATGAT-3′ (antisense); Imd, 5′-CCCTTCCTTCCAGGCTTAG-3′ (sense) and 5′-AGGCGATTGGCTAAGATTCTG-3′ (antisense); Il-12, 5′-CTGTTGCGTTGTTAGTACCTCTAT-3′ (sense) and 5′-GCCAGTGTCGCCATTAGATTC-3′ (antisense); Il-10, 5′-GCTTCTACT-GACTGGCATGAG-3′ (sense) and 5′-CCGACGTCTTAGGACCATGTTG-3′ (antisense); inos, 5′-GGAGTGACGGGCCAAC-ATGACT-3′ (sense) and 5′-TCGATGCAACAACGTTGGTAAAC-3′ (antisense); and Arg1, 5′-TTTTTCCAGCAGACCCAGCTT-3′ (sense) and 5′-AGAGATTATCGGAGCCGCCT-3′ (antisense). Cell or mouse tissue extracts containing equal amounts of total protein were resolved by 10% SDS-PAGE (15% SDS-PAGE for IMD) for Western blotting analysis. The blots were incubated with primary antibodies and IRDye 700DX-conjugated secondary antibodies (Rockland Inc.). For the primary antibody dilution, 1:500 dilution was used for iNOS, and 1:1000 dilutions were used for the other antibodies. The immunofluorescence signal was detected by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE).

**siRNA for AMPK**—AMPK siRNA was designed and chemically modified by the manufacturer (Ribobio). Sequences corresponding to the siRNA of AMPK were as follows: sense, 5′-GCAGAAGAUUCCGAGCUCU dTdT-3′; antisense, 3′-dTdT CAGUCUUCUAGCCUCGAA-5′. Transfection of mouse peritoneal macrophages with the siRNA (50 nmol/l in vitro) was performed with Oligofectamine (Invitrogen) according to the instructions of the manufacturer.

**Flow Cytometry**—Adipose tissues were minced and digested with 1 mg/ml type I collagenase for 40 min. After centrifugation, stromal vascular fractions were collected at the bottom of the tubes. Then the stromal vascular fractions were stained with the macrophage marker F4/80 allolymphocyanin, the dead cell marker 7-aminoactinomycin D, the M2 marker CD206, the M1 marker CD11c, the M1 marker CD11c phycoerythrin (BD Biosciences) and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Cell Culture**—Mouse peritoneal macrophages were cultured with 300 μM Hcy, 20 nM IMD, or Hcy + IMD for 24 h in DMEM (Invitrogen) supplemented with 2% FBS. Cells were then used for M1/M2 marker or pAMPK detection. For the co-culture assay, the cells were changed to fresh medium for an additional 24 h. Subsequently, cell culture supernatants were collected. 3T3-L1 cells were cultured in growth medium, high-glucose DMEM, supplemented with 10% FBS. Two days post-confluence, cells were induced to differentiate with a standard mixture consisting of growth medium with 1 μmol/liter dexamethasone, 10 μg/ml bovine insulin, and 0.25 mmol/liter 3-isobutyl-1-methylxanthine. After 3 days in differentiation medium, cells were treated with growth medium with 10 μg/ml bovine insulin for 3 days and then maintained in growth medium alone. Cells were considered mature adipocytes 8 days post-induction of differentiation and then treated with supernatant from cultured macrophages for 24 h.
IMD Restores HHcy-induced Insulin Resistance

**A**

FIGURE 1. IMD expression was inhibited in HHcy mice. A, plasma samples were collected from HHcy or age-matched control (Ctl) mice for an IMD ELISA assay (n = 3 for controls; n = 4 for HHcy). B, mRNA expression of IMD was detected in eWAT, livers, and skeletal muscles of HHcy and control mice by real-time PCR (n = 4). C, Western blotting analysis of IMD expression in eWAT. Quantitative analysis is shown in the right panel (n = 3/group). N.D., not detected; *, p < 0.05; **, p < 0.01.

Statistical Analysis—All data are reported as mean ± S.E. Data were analyzed with GraphPad Prism software. Statistical analysis involved one-way analysis of variance for multiple comparisons, then Tukey-Kramer post-hoc testing, and Student’s unpaired t test for comparisons between two groups. p < 0.05 was considered statistically significant.

**Results**

**IMD Expression Was Decreased in HHcy Mice**—After being fed with 1.8 g/liter Hcy in the drinking water for 4 weeks, HHcy mice displayed insulin resistance (23). To validate whether IMD plays a role in HHcy-induced insulin resistance, we detected the expression level of IMD in HHcy mice. Compared with controls, the plasma IMD levels of HHcy mice were markedly lower (Fig. 1A). IMD was abundant in adipose tissues compared with livers or skeletal muscles (Fig. 1B). Thus, we proposed that adipose tissue is the pivotal organ where IMD participates in glucose metabolism. Consistent with systemic IMD down-regulation, the mRNA and protein levels of IMD were apparently decreased in the adipose tissues of HHcy mice (Fig. 1, A and B).

**IMD Reversed HHcy-induced Insulin Resistance in Adipose Tissue**—To investigate the role of adipocyte IMD in HHcy-mediated insulin resistance, we generated adipose tissue (Fabp4)–specific knockin IMD gene (Adipo-IMD-tg) mice. Adipo-IMD-tg mice showed an accurate transgenic gene band, but wild-type mice did not (data not shown). The mRNA levels of IMD were substantially increased in epididymal white adipose tissues (eWAT) but not in livers or skeletal muscles (Fig. 2A). It was reported that Fabp4 is expressed in macrophages (27). Therefore, the expression of the IMD transgene in macrophages was also investigated. Adipo-IMD-tg mice exhibited significantly enhanced protein levels of IMD in eWAT, whereas no changes in IMD expression in macrophages were detected (Fig. 2B). The body weights of Adipo-IMD-tg mice were similar to those of controls after feeding with Hcy for 4 weeks (Fig. 2C). The fat-to-weight ratio in Adipo-IMD-tg mice was similar to those of controls (Fig. 2D). No difference was observed in the size of adipocytes in eWAT tissues from Adipo-IMD-tg mice and control mice (Fig. 2E). Consistent with our data published previously (23), HHcy mice had impaired insulin sensitivity, as verified by GTT and ITT (data not shown). However, the GTT and ITT results indicated improved glucose tolerance and insulin tolerance, respectively, in Adipo-IMD-tg mice with HHcy (Fig. 2F). Moreover, fasted serum insulin levels and the HOMA index were significantly reduced in Adipo-IMD-tg mice (Fig. 2G). The insulin sensitivity indicator phosphorylated AKT (Ser-473) was reduced in adipose tissues of HHcy mice but elevated in the Adipo-IMD-tg mouse group (Fig. 2H). These results indicated that IMD overexpression ameliorates systemic and adipose tissue insulin resistance in HHcy mice.

**IMD Attenuated Hcy-induced M1/M2 Imbalance**—To further explore the mechanisms underlying IMD-ameliorated insulin resistance with HHcy, the balance of pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages was detected in the adipose tissues of Adipo-IMD-tg mice. In a flow cytometry assay gated on F4/80-positive macrophages, CD11c was labeled as M1, whereas CD206 was labeled as an M2 marker (28). In eWAT, an increased proportion of M2 macrophages was observed in Adipo-IMD-tg mice (Fig. 3A). Moreover, a reduced percentage of M1 macrophages was observed in subcutaneous white adipose tissues (Fig. 3B). This imbalance of M1/M2 was further confirmed by examination of cell surface markers (CD11C for M1 and CD206 for M2), cytokines (IL-12 for M1 and IL-10 for M2), and enzymes (iNOS for M1 and Arg1 for M2). Elevated mRNA expression of M2 markers, including Cx206, IL-10, and Arg1, and reduced mRNA expression of iNos were observed in the eWAT of Adipo-IMD-tg mice compared with control mice (Fig. 3C). To verify the effect of IMD on the M1/M2 cell balance in vitro, thioglycolate-elicited peritoneal macrophages were administered (300 μM Hcy, 20 nM IMD, or 300 μM Hcy + 20 nM IMD) for 24 h. The expressions of M1 and M2 macrophage markers were detected. The administration of IMD reversed Hcy-induced M1 macrophage marker expression (i.e. Cd11c and iNos; Fig. 4, A, C, and E), whereas it recovered Hcy-inhibited M2 macrophage marker expression (i.e. Cd206, IL-10, and Arg1; Fig. 4, A, B, D, and E). These data indicate that IMD improves the Hcy-induced M1/M2 imbalance.

**IMD Restored the Hcy-induced M1/M2 Imbalance by Activating the AMPK Pathway**—AMP-activated protein kinase (AMPK) is a key regulator of cellular and systemic energy homeostasis. It has recently been reported that the depletion of AMPK in hematopoietic cells inhibits M2 macrophages but promotes M1 macrophage activation (29). The AMPK pathway was further detected in macrophages treated with Hcy and IMD alone or in combination. The phosphorylation of AMPKα was inhibited by Hcy treatment but was reversed by IMD in macro-
To address the effect of AMPK on Hcy- or IMD-mediated macrophage polarization, the activity of AMPK was abolished by siRNA. Around half of the AMPK expression was knocked down by AMPK siRNA (Fig. 5B). Although IMD reversed the Hcy-induced M1/M2 imbalance, knockdown of AMPK diminished these effects. Moreover, the protein expression of ARG1 was markedly reduced, whereas iNOS expression was enhanced by siAMPK transfection (Fig. 5, C and D). These results suggest that IMD reverses the Hcy-induced M1/M2 imbalance through the AMPK pathway.

**IMD Reversed HHcy-induced ER Stress and Chronic Inflammation in Adipose Tissue—**Previous studies from our group showed that HHcy enhances ER stress and insulin resistance in adipocytes. The PERK and ATF6 pathways were involved rather than the XBP1 pathway (23). To address the effect of IMD on HHcy-primed ER stress, adipose tissues from Adipo-IMD-tg mice were analyzed regarding the PERK and ATF6 signaling pathways. Compared with controls, Adipo-IMD-tg mice displayed reduced phosphorylated PERK (pPERK) and phosphorylated eIF2α (pelF2α) levels in adipose tissues with HHcy.

**FIGURE 2.** IMD improved HHcy-induced insulin resistance in adipose tissues. A, mRNA expression of IMD in eWAT, livers, and skeletal muscles (SK) of Adipo-IMD-tg and control (Ctl) mice (n = 4). B, protein expression of IMD in eWAT (top panel) and peritoneal macrophages (bottom panel) by Western blotting analysis. C, body weights of controls and Adipo-IMD-tg mice (n = 6, 8/group). D, MRI analysis of fat in Adipo-IMD-tg mice. E, H&E staining of eWAT from control and Adipo-IMD-tg mice (n = 4 for controls; n = 5 for Adipo-IMD-tg mice). F, GTT and ITT were examined in Adipo-IMD-tg mice (n = 4–5/group). G, fasting glucose, fasting serum insulin levels, and HOMA index of controls or Adipo-IMD-tg mice with HHcy (n = 4 and n = 5 mice for each group, respectively). H, phosphorylated AKT (Ser-473) was detected in eWAT. The expression of pAKT was quantified by total AKT, as shown in the right panel (n = 3/group). N.D., not detected; *, p < 0.05; **, p < 0.01; ***, p < 0.001.)
Activated/cleaved ATF6 was also repressed in adipose tissues of Adipo-IMD-tg mice (Fig. 6, A and B). Our results suggest that, under the HHcy condition, overexpression of IMD attenuates the ER stress pathways in adipose tissues.

Inflammation promotes HHcy-induced insulin resistance in adipocytes and triggers the activation of JNK, which is downstream of ERS in adipocytes (23). The phosphorylation levels of JNK in the adipose tissues of Adipo-IMD-tg mice were substantially reduced compared with those of wild-type mice under HHcy (Fig. 6, C and D). The activation of the NF-κB P65 subunit was significantly inhibited in Adipo-IMD-tg mice (Fig. 6, C and D). IMD ameliorates HHcy-aggravated inflammation in adipose tissue.

**IMD Reversed Hcy-induced ER Stress and Inflammation in Adipocytes as a Result of Improvement in Macrophage Polarization**—In adipose tissues, macrophage polarization is a well known inflammation mediator of cross-talk with adipocytes and interferes with adipocyte insulin signaling (13, 14, 25). Therefore, whether IMD- or Hcy-mediated M1/M2 imbalance interacts with ER stress and inflammation in adipocytes was investigated by co-culture studies of macrophages and adipocytes in vitro. Mouse peritoneal macrophages were treated with Hcy, IMD, or Hcy + IMD. Cell culture supernatants were added to the culture of differentiated 3T3-L1 adipocytes. PERK and ATF6 signaling pathways were detected in the treated adipocytes. Supernatant from Hcy-treated macrophages enhanced the levels of pPERK, peIF2α, and cleaved ATF6 in adipocytes, whereas the Hcy + IMD group reversed these effects (Fig. 7A). IMD reversed the Hcy-induced activation of JNK (Fig. 7B). Collectively, these data indicate that soluble factors in the macrophage culture supernatants mediated the IMD reversal of Hcy-induced ER stress and chronic inflammation in adipocytes.

**IMD Treatment Improved Hcy-induced Insulin Resistance**—To verify the therapeutic effect of IMD on Hcy-induced insulin resistance, we implanted osmotic minipumps containing IMD subcutaneously to elevate the IMD level in HHcy mice. GTT and ITT showed improved glucose intolerance and increased insulin sensitivity in IMD-treated mice (Fig. 8A), whereas the body weight remained similar in the two groups (Fig. 8B). The fasted serum insulin levels and HOMA indexes were significantly reduced in the IMD treatment group (Fig. 8C). In adipose tissues, phosphorylated AKT (Ser-473) was elevated by IMD infusion compared with controls with HHcy (Fig. 8D). These results reveal that IMD administration produced striking benefits for the improvement of HHcy-induced insulin resistance.

**Discussion**

Our previous study reported that HHcy induces ERS and insulin resistance in adipose tissues (23). In this study, adipocyte-specific IMD gene overexpression or the administration of IMD was shown to ameliorate HHcy-induced insulin resistance. Unlike enhancing thermogenesis in brown adipose tissues of high-fat diet-induced obese mice (12), IMD displayed a
distinct role in HHcy-induced insulin resistance. Mechanism studies revealed that Hcy enhances the ratio of M1/M2 by repression of AMPK activity and that IMD administration reverses the effect of Hcy on AMPK activity and M1/M2 balance. The restoration of macrophage polarization contributed to the IMD improvement of Hcy-induced ER stress and chronic inflammation in adipocytes (Fig. 8E).

Inflammatory signals such as JNK activation inhibit the phosphorylation of insulin receptor substrate, which results in insulin resistance (15, 30). It has been reported that IMD attenuates pro-inflammatory cytokine expression, including TNFα, IL-6, and IL-1β, in the testis in a LPS-induced rat orchitis model (31). The reduction of IMD expression in the skin of atopic dermatitis patients compared with healthy controls indicated that lower levels of IMD are a higher susceptibility factor to inflammatory stimuli (16). This study consistently demonstrated that IMD-specific overexpression in adipose tissues reversed HHcy-induced inflammation and insulin resistance. Although some reports have shown that Fabp4 was expressed in macrophages, no significant expression of transgenic IMD was induced in
macrophages compared with adipocytes in Adipo-IMD-tg mice. Supporting our findings, a Fabp4-PPARγ overexpression line demonstrated that the transgenic PPARγ was specifically expressed in adipocytes (32), and different Fabp4-driven Cre mouse models showed that the floxed alleles were recombined in adipocytes but not in macrophages (33).

Increasing infiltration of pro-inflammatory immune cells, particularly macrophages, is a characteristic of obesity-induced insulin resistance (25, 34). Insulin resistance is promoted by a transition in macrophage polarization from an anti-inflammatory M2 activation state to an inflammatory M1 activation state (14, 35–37). Previous reports have indicated that HHcy promotes the differentiation of Ly6C-high inflammatory monocytes in an atherosclerosis mouse model (19). Another recent study demonstrated that Hcy induces M1 polarization and converts M2 to an M1 subtype in vitro (26). In this study, IMD overexpression reversed the

**FIGURE 5. IMD reversed the Hcy-induced M1/M2 imbalance through the AMPK pathway.** A, after being treated with Hcy, IMD, or Hcy + IMD, peritoneal macrophage cells were analyzed for the expression of phosphorylated AMPKα. Quantitative data, shown in the right panel, are from three independent experiments. Ctl, control. B, the expression of AMPK was knocked down by siRNA. The knockdown efficiency was verified by real-time PCR. C and D, Arg1 (C) and iNOS (D) expression was detected by Western blotting assay. Quantitative data in the bottom panels are results from three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
ratio of M1/M2 under the HHcy condition both in vivo and in vitro. These findings uncovered the mechanisms of IMD-inhibited inflammation in adipose tissues.

AMPK activation is considered to be a potential therapeutic target for insulin resistance. Activated AMPK stimulates glucose uptake in skeletal muscles and fatty acid oxidation in adipose tissues, and it reduces hepatic glucose production (38, 39). The inactivation of macrophage AMPK inhibits adipocyte insulin signaling and glucose uptake in a macrophage-adipocyte co-culture system (40). AMPK activation is related to repressed iNOS in primary human umbilical vein endothelial cells, myocytes, macrophages, and adipocytes (41, 42). AMPKα1−/− macrophages did not acquire the phenotype or the functions of M2 macrophages (43). In agreement with these findings, this study demonstrated that Hcy administration repressed the activation of AMPK but that IMD treatment reversed the effect of Hcy on AMPK activity and the M1/M2 balance. Blockage of AMPK enhanced M1 macrophage marker iNOS expression but repressed M2 macrophage marker ARG1 expression. Our data provided a new mechanism for Hcy and IMD in the regulation of the M1/M2 balance. Unlike other members of the calcitonin gene-related peptide/calcitonin family, no unique receptor for IMD has been identified. Intermedin binds nonselectively to both calcitonin gene-related peptide and adrenomedullin (ADM) receptor complexes: CRLR-RAMP1, 2, and 3 (44). IMD couples with the CRLR-RAMP receptor complexes to activate cAMP production (45). cAMP is known to regulate AMPK activity (46). IMD may be signaling, through CRLR-RAMP receptors, to activate AMPK through cAMP in macrophages. Further studies are needed to clarify this issue.

In white adipose tissues, the metabolic functions are regulated by cross-talk between adipocytes and stromal cells, particularly with macrophages (47). Pro-inflammatory M1 macrophages inhibit insulin sensitivity by cytokine production, whereas anti-inflammatory M2 macrophages reverse the effect of M1 macrophages (36). Our data indicated that IMD improved the M1/M2 imbalance induced by Hcy. The distinct soluble factors produced from Hcy and/or IMD treatment led to ERS in adipocytes. Our findings revealed a possible interaction between macrophages and adipocytes in IMD- and/or HHcy-treated mice.

Our study demonstrated that IMD reverses HHcy-induced ERS, inflammation, and insulin resistance. Hcy elevated the
M1/M2 ratio by inhibiting AMPK activity, and IMD reversed the M1/M2 imbalance. This study revealed the role of IMD administration in the recovery of insulin sensitivity, which shed light on the molecular mechanisms of Hcy and IMD regulation of the M1/M2 balance, and it provided a new therapeutic strategy for insulin resistance.

Author Contributions—Y. P., Yang Li, Y. Lv, L. S., S. Z., Y. W., Yin Li, G. L., M. X., X. W., and C. J. made substantial contributions to the acquisition of data or the analysis and interpretation of data. Y. P., C. J., and X. W. contributed to the design of the experiments and the writing of the manuscript. All authors approved the final version of the paper. X. W. and C. J. are the guarantors of this work and, as such, had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Roh, J., Chang, C. L., Bhalla, A., Klein, C., and Hsu, S. Y. (2004) Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J. Biol. Chem. 279, 7264–7274
2. Takei, Y., Hyodo, S., Katafuchi, T., and Minamino, N. (2004) Novel fish-derived adrenomedullin in mammals: structure and possible function. Peptides 25, 1643–1656
3. Li, Y., Jiang, C., Wang, X., Zhang, Y., Shibahara, S., and Takahashi, K. (2007) Adrenomedullin is a novel adipokine: adrenomedullin in adipocytes and adipose tissues. Peptides 28, 1129–1143
4. Nomura, I., Kato, J., Tokashiki, M., and Kitamura, K. (2009) Increased plasma levels of the mature and intermediate forms of adrenomedullin in obesity. Regul. Pept. 158, 127–131
5. Shimosawa, T., Oghira, T., Matsui, H., Asano, T., Ando, K., and Fujita, T. (2003) Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. Hypertension 41, 1080–1085
6. Zhao, L., Peng, D. Q., Zhang, J., Song, J. Q., Teng, X., Yu, Y. R., Tang, C. S., and Qi, Y. F. (2012) Extracellular signal-regulated kinase 1/2 activation is involved in intermedin1–53 attenuating myocardial oxidative stress injury induced by ischemia/reperfusion. Peptides 33, 329–335
7. Bell, D., and McDermott, B. J. (2008) Intermedin (adrenomedullin-2): a novel counter-regulatory peptide in the cardiovascular and renal systems. Br. J. Pharmacol. 153, S247–262
8. Li, H., Bian, Y., Zhang, N., Guo, J., Wang, C., Lau, W. B., and Xiao, C. (2013) Intermedin protects against myocardial ischemia-reperfusion injury in diabetic rats. Cardiovasc. Diabetol. 12, 91
9. Qiao, X., Li, R. S., Li, H., Zhu, G. Z., Huang, X. G., Shao, S., and Bai, B. (2013) Intermedin protects against renal ischemia-reperfusion injury by inhibition of oxidative stress. Am. J. Physiol. Renal Physiol. 304, F112–119
10. Dai, X. Y., Cai, Y., Mao, D. D., Qi, Y. F., Tang, C., Xu, Q., Zhi, Y., Xu, M. J., and Wang, X. (2012) Increased stability of phosphatase and tensin homolog by intermedin leading to scavenger receptor A inhibition of macrophages reduces atherosclerosis in apolipoprotein E-deficient mice. J. Mol. Cell. Cardiol. 53, 509–520
11. Dai, X. Y., Cai, Y., Sun, W., Ding, Y., Wang, W., Kong, W., Tang, C., Zhu, Y., Xu, M. J., and Wang, X. (2014) Intermedin inhibits macrophage foam-cell formation via triptetraprolin-mediated decay of CD36 mRNA. Cardiovasc. Res. 101, 297–305
12. Zhang, H., Zhang, S. Y., Jiang, C., Li, Y., Xu, G., Xu, M. J., and Wang, X. (2016) Intermedin/adrenomedullin 2 polypeptide promotes adipose tissue browning and reduces high-fat diet-induced obesity and insulin resistance in mice. Int. J. Obes. (Lond.) 40, 852–860
13. Heilbronn, L. K., and Campbell, L. V. (2008) Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Curr. Pharm. Des. 14, 1225–1230
14. Olesfsky, J. M., and Glass, K. C. (2012) Macrophages, inflammation, and insulin resistance. Annua. Rev. Physiol. 72, 219–246
15. Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature 444, 860 – 867
16. Kindt, F., Wiegand, S., Löser, C., Nilles, M., Niermeier, V., Hsu, S. Y., Steinhoff, M., Kummer, W., Gieler, U., and Haberberger, R. V. (2007) Intermedin: a skin peptide that is downregulated in atopic dermatitis. J. Invest. Dermatol. 127, 605–613
17. Zeng, X., Dai, J., Remick, D. G., and Wang, X. (2003) Homocysteine mediated expression and secretion of monocyte chemotactant protein-1 and interleukin-8 in human monocytes. Circ. Res. 93, 311–320
18. Zhang, D., Jiang, X., Fang, P., Yan, Y., Song, J., Gupta, S., Schafer, A., Durante, W., Kruger, W. D., Yang, X., and Wang, H. (2009) Hyperhomocysteinemia promotes inflammatory monocyte generation and accelerates atherosclerosis in transgenic cystathionine β-synthase-deficient mice. Circulation 120, 1893–1902
19. Zhang, D., Fang, P., Jiang, X., Nelson, J., Moore, I. K., Kruger, W. D., Berretta, R. M., Houser, S. R., Yang, X., and Wang, H. (2012) Severe hyperhomocysteinemia promotes bone marrow-derived and resident inflammatory macrophage differentiation and atherosclerosis in LDLr/CBS-deficient mice. Circ. Res. 111, 37–49
20. Dai, J., Li, W., Chang, L., Zhang, Z., Tang, C., Wang, N., Zhu, Y., and Wang, X. (2006) Role of redox factor-1 in hyperhomocysteinemia-accelerated atherosclerosis. Free Radic. Biol. Med. 41, 1566–1577
21. Huang, T., Ren, J., Huang, J., and Li, D. (2013) Association of homocysteine with type 2 diabetes: a meta-analysis implementing Mendelian randomization approach. BMC Genomics 14, 867
22. Li, Y., Jiang, C., Xu, G., Wang, N., Zhu, Y., Tang, C., and Wang, X. (2008) Homocysteine upregulates resistin production from adipocytes in vivo and in vitro. Diabetes 57, 817–827
23. Li, Y., Zhang, H., Jiang, C., Xu, M., Pang, Y., Feng, J., Xiang, X., Kong, W., Xu, G., Li, Y., and Wang, X. (2013) Hyperhomocysteinemia promotes insulin resistance by inducing endoplasmic reticulum stress in adipose tissue. J. Biol. Chem. 288, 9583–9592
24. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Ferrante, A. W., Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest. 112, 1796–1808
25. Fernández-Real, J. M., and Pickup, J. C. (2012) Innate immunity, insulin resistance and type 2 diabetes. Diabetologia 55, 273–278
26. Gao, S., Wang, L., Liu, W., Wu, Y., and Yuan, Z. (2014) The synergistic effect of homocysteine and lipopolysaccharide on the differentiation and conversion of raw264.7 macrophages. J. Inflamm. (Lond.) 11, 13
27. Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., Parker, R. A., Suttles, J., Fazio, S., Hotamisligil, G. S., and Linton, M. F. (2001) Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. Nat. Med. 7, 699–705
28. Fujisaka, S., Usui, I., Bukhari, A., Ikutani, M., Oya, T., Kanatani, Y., Tsuneyama, K., Nagai, Y., Takats, K., Urakaze, M., Leibel, R. L., and Tobe, K. (2009) Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. Diabetes 58, 2574–2582
29. Galic, S., Fullerton, M. D., Schertzer, J. D., Siddiqua, S., Marcinko, K., Walkley, C. R., Ison, D., Honeyman, J., Chen, Z. P., van Denderen, B. J., Kemp, B. E., and Steinberg, G. R. (2011) Hematopoietic AMPKβ1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. J. Clin. Invest. 121, 4903–4915
30. Copp, K. D., and White, M. F. (2012) Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 55, 2565–2582
31. Li, L., Ma, P., Liu, Y., Huang, C., O., W., S., Tang, F., and Zhang, J. V. (2013) Intermedin attenuates LPS-induced inflammation in the rat testis. PLoS ONE 8, e65278
32. Sugii, S., Olson, P., Sears, D. D., Saberi, M., Atkins, A. R., Barish, G. D., Hong, S. H., Castro, G. L., Yin, Y. Q., Nelson, M. C., Hsiao, G., Greaves, D. R., Downes, M., Yu, R. T., Olefsky, J. M., and Evans, R. M. (2009) PPARγ activation in adipocytes is sufficient for systemic insulin sensitization. Proc. Natl. Acad. Sci. U.S.A. 106, 22504–22509
33. Lee, Y. S., Kim, J. W., Osborne, O., Oh, M., Sasaki, S., Schenk, S., Chen, A., Chung, H., Murphy, A., Watkins, S. M., Quehenberger, O., Johnson, R. S., and Olefsky, J. M. (2014) Increased adipocyte O2 consumption triggers HIF-1α, causing inflammation and insulin resistance in obesity. Cell 157, 1339–1352
34. Sell, H., Habich, C., and Eckel, J. (2012) Adaptive immunity in obesity and insulin resistance. Nat. Rev. Endocrinol. 8, 709–716
35. Kraakman, M. J., Murphy, A. J., Landevelt-Dahm, K., and Kammoun, H. L. (2014) Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function? Front. Immunol. 5, 470
36. Luneng, C. N., Bodzin, J. L., and Saltiel, A. R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J. Clin. Invest. 117, 175–184
37. Fadini, G. P., de Kreutzenberg, S. V., Boscaro, M., Cappellari, R., Kräkel, N., Landmesser, U., Toniolo, A., Bolego, C., Cignarella, A., Seeger, F., Dimmelser, S., Zeiher, A., Agostini, C., and Avogaro, A. (2013) An unbalanced monocyte polarization in peripheral blood and bone marrow of patients with type 2 diabetes has an impact on microangiopathy. Diabetologia 56, 1856–1866
38. Steinberg, G. R., and Kemp, B. E. (2009) AMPK in health and disease. Physiol. Rev. 89, 1025–1078
39. Ruderman, N. B., Carling, D., Prentki, M., and Cacicedo, J. M. (2013) AMPK, insulin resistance, and the metabolic syndrome. J. Clin. Invest. 123, 2764–2772
40. Yang, Z., Kahn, B. B., Shi, H., and Xue, B. Z. (2010) Macrophase α1 AMP-activated protein kinase (α1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. J. Biol. Chem. 285, 19051–19059
41. Lee, C. H., Lee, S. D., Ou, H. C., Lai, S. C., and Cheng, Y. J. (2014) Eicosapentaenoic acid protects against palmitic acid-induced endothelial dysfunction via activation of the AMPK/eNOS pathway. Int. J. Mol. Sci. 15, 10334–10349
42. Pilon, G., Dallaire, P., and Mareette, A. (2004) Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase: a new mechanism of action of insulin-sensitizing drugs. J. Biol. Chem. 279, 20767–20774
43. Mounier, R., Théret, M., Arnold, L., Cuvellier, S., Baltot, L., Göransson, O., Sanz, N., Ferry, A., Sakamoto, K., Foretz, M., Violette, B., and Chazaud, B. (2013) AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. Cell Metab. 18, 251–264
44. Hong, Y., Hay, D. L., Quirion, R., and Poyner, D. R. (2012) The pharmacology of adrenomedullin 2/intermedin. Br. J. Pharmacol. 166, 110–120
45. Chang, C. L., Roh, J., and Hsu, S. Y. (2004) Intermedin, a novel calcitonin family peptide that exists in teleosts as well as in mammals: a comparison of other calcitonin/intermedin family peptides in vertebrates. Peptides 25, 1633–1642
46. Omar, B., Zmuda-Trzebiatowska, E., Manganiello, V., Göransson, O., and Degerman, E. (2009) Regulation of AMP-activated protein kinase by cAMP in adipocytes: roles for phosphodiesterases, protein kinase B, protein kinase A, Epac and lipolysis. Cell. Signal. 21, 760–766
47. Wynn, T. A., Chawla, A., and Pollard, J. W. (2013) Macrophage biology in development, homeostasis and disease. Nature 496, 445–455