antibodies (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Images of the pancreatic tissue and islet β-cells were viewed on the monitor of a computer through a microscope connected to a camera with a charged-coupled device (Olympus, Tokyo, Japan). The areas of the pancreata and β-cells were traced manually and analyzed with Win ROOF software (Mitani, Chiba, Japan), as previously described (15). At least 50 islets per mouse were analyzed. BrdU incorporation was analyzed as described previously (16). In brief, BrdU (100 mg/kg in saline; Sigma-Aldrich, St. Louis, MO) was injected intraperitoneally, and the pancreas was removed 6 hours later. The sections were immunostained with anti-BrdU antibody (diluted 1:200; Dako, Glostrup, Denmark). BrdU-positive β-cells were quantitatively assessed as a percentage of the total number of β-cells by counting the cells in a minimum of 50 islets per mouse. Immunohistochemical staining for pimonidazole were carried out as described previously (17), with slight modifications. The oxygenation marker pimonidazole was injected intravenously into the tail vein (60 mg/kg). Two hours later, the animals were killed and their pancreata were removed and prepared for histological analysis. The sections were immunostained with anti-pimonidazole antibody (diluted 1:100; Hypoxyprobe, Burlington, MA). Pimonidazole-positive islets were quantitatively assessed as a percentage of the total number of islets by counting in a minimum of 50 islets per mouse.

Perfusion experiments using mouse pancreata

Mice were used for the perfusion experiments after they had been denied access to food overnight for 16 h, as previously reported (18) with slight modifications. Briefly, the superior mesenteric and renal arteries were ligated, and the aorta was tied just below the diaphragm. The perfusate was infused via a catheter placed in the aorta and collected

from the portal vein. The perfusate used was KRB HEPES buffer supplemented with 4.6% dextran and 0.25% BSA and gassed with 95% O_2 / 5% CO_2. The flow rate of the perfusate was 1 mL/min. Pancreata were perfused with KRB HEPES buffer containing 2.8 or 22.2 mmol/L glucose. The perfusion protocols began with a 10-min equilibration period with the same buffer used in the initial step (i.e., from 1 to 5 min), as shown in the figures. The insulin levels in the perfusate were measured using an RIA kit.

Islet blood flow measurements

The experiments were performed according to a protocol described in detail in a previous report (19). Briefly, polyethylene catheters were inserted via the right carotid artery into the ascending aorta and into the femoral artery. After the blood pressure stabilized, nonradioactive microspheres (Dye-Trak; Triton Technology, Los Angeles, CA) with a mean diameter of 10 μm were injected for 10 s via the catheter placed with its tip in the ascending aorta. Starting 5 s before the microsphere injection and continuing for a total of 60 s, an arterial blood sample was collected from the catheter in the femoral artery at a rate of ~0.50 mL/min. The exact withdrawal rate was confirmed in each animal by weighing the sample. After the reference sample was obtained, another blood sample was drawn for the measurement of the blood glucose level. The whole pancreata were removed, blotted, weighed, and treated using a freeze-thawing technique to visualize the microspheres. The capillary blood flow values were calculated according to the formula Qorg = Qref x Norg/Nref, where Qorg is the organ capillary blood flow (mL/min), Qref is the withdrawal rate of the reference sample (mL/min), Norg is the number of microspheres present in the organ, and Nref is the number of microspheres in the reference sample. The microsphere contents of the adrenal glands were used as a control to confirm that the microspheres had adequately

mixed in the arterial circulation (19). A <10% difference in the numbers of microspheres between the right and left adrenal glands was taken to indicate sufficient mixing. The islet blood flow was expressed per islet weight estimated by multiplying the pancreatic weight with the islet volume fraction of the whole pancreas in each animal. To evaluate the effect of insulin on the islet blood flow, insulin (0.75 U/kg body weight) was injected intraperitoneally as described previously (20) with slight modifications. The anaesthetised animals were left for 20 minutes. Before microsphere injection, blood glucose levels were determined using arterial blood samples.

Lectin perfusion and vascular staining

Fluorescein-labeled lectin (Lycopersicon Esculentum; Vector, Buringame, CA), which binds specifically to endothelial cells and epithelial cells, were injected into the tail vein (1 mg/mL solution/0.1 mL/mouse) and were allowed to circulate for 3 min. The pancreas was then excised and immersion-fixed in 4% paraformaldehyde for 16 h at 4°C. After fixation, the pancreata were immersed in 30% sucrose as a cryoprotectant, after which the tissues were embedded in optimal cutting temperature compound (SAKURA SEIKI, Tokyo, Japan). The block was sectioned into 15-μm-thick sections and collected on microscope slides. Images of the vasculature were viewed on the monitor of a computer through a CCD camera with Biozero (KEYENCE, Osaka, Japan). The areas of the vasculature and β-cells were traced manually and analyzed with a software program (Image J). At least 50 islets were analyzed per mouse.

Statistical analysis

Values were expressed as the mean ± SE. The statistical significance of differences between groups was determined using a 2-tailed indirect Student t-test. Data involving more than two groups were assessed using an analysis of variance (ANOVA).

RESULTS

Insulin secretion in response to glucose, glucagon, and arginine was impaired in the ETIrs2KO mice

The blood glucose levels at 5 and 15 min after glucose loading were significantly higher in the ETIrs2KO mice than in the control mice at 12 weeks (Fig. 1*A*). The plasma insulin levels at 2 and 5 min were significantly lower in the ETIrs2KO mice than in the control mice at 12 weeks (Fig. 1*A*). At 24 weeks, although the blood glucose level at 5 min was comparable to that in the control mice, the blood glucose level at 15 min was significantly higher in the ETIrs2KO mice (Fig. 1*B*). The plasma insulin levels at 2 min after glucose loading were significantly lower in the ETIrs2KO mice (Fig. 1*B*). In addition to glucose, glucagon-induced and arginine-induced insulin secretion were also significantly impaired in the ETIrs2KO mice (Fig. 1*C*, *D*). These findings suggest that insulin secretion induced by various secretagogues is impaired in the ETIrs2KO mice.

Insulin secretion in isolated islets was not impaired in the ETIrs2KO mice

To clarify the molecular mechanism responsible for the impairment of insulin secretion in the ETIrs2KO mice, we next measured insulin secretion from isolated islets using static and perifusion analyses at 12 weeks. In contrast to the results of the in vivo study, glucose-stimulated insulin secretion was comparable between the control and the ETIrs2KO mice in a static incubation experiment (Fig. 2*A*). Moreover, the perifusion experiments also demonstrated that insulin secretion under perifusion with a stimulating glucose concentration was comparable between the control and the ETIrs2KO mice at 12 weeks (Fig. 2*B*). These data indicated that insulin secretion from isolated islets was not impaired in the ETIrs2KO mice. The gene expression levels of Insulin1 and Insulin2

(Fig. 2*C*) and the insulin contents (Fig. 2*D*) in the isolated islets were not significantly different between the control and the ETIrs2KO mice at 12 weeks. Consistent with our previous study (14), the β-cell mass from the ETIrs2KO mice tended to be larger, but no statistical difference was seen between the two groups (Fig. 2*E*, *F*) at 24 weeks. In addition, the rate of BrdU incorporation into the pancreatic β-cell nuclei in the ETIrs2KO mice tended to be increased but was not significantly different, compared with that in the control mice at 24 weeks (Fig. 2*G*). These data suggest that the impairment of β-cell function and/or mass often lead to impaired insulin secretion, which was not observed in the ETIrs2KO mice.

Insulin secretion was significantly decreased in the ETIrs2KO mice during pancreatic perfusion

The impairment of insulin secretion observed in vivo, but not in vitro, prompted us to investigate the secretory responses of the pancreas in perfusion experiments that were capable of assessing insulin secretion via blood vessels. The insulin responses to glucose were significantly impaired in the ETIrs2KO mice pancreata at 12 weeks (Fig. 3*A*). In addition, the amount of secreted insulin (AUCinsulin) after glucose stimulation (from 5 to 30 min) was also significantly impaired in the ETIrs2KO mice (Fig. 3*B*). These data suggest that the impaired insulin secretion in the ETIrs2KO mice is caused by an impairment in the blood circulation, and not the dysfunction of the β- cells.

The islet blood flow was significantly decreased in the ETIrs2KO mice

The mean blood pressure in anesthetized mice was similar between the control and the ETIrs2KO mice at 9-12 weeks (Fig. 4*A*). Although there was no significant difference in the pancreatic blood flow (Fig. 4*B*), the number of microspheres in islets and islet blood flow were significantly decreased in the ETIrs2KO mice, compared with that in

the control mice in the basal state (Fig. 4*C*, *D*). Moreover, in the insulin-treated state, islet blood flow was significantly decreased in the ETIrs2KO mice at 9 weeks (Fig. 4*E*, Table 1). These findings suggest that the absence of Irs2 in endothelial cells induce a reduction in the islet blood flow, similar to previous observations in skeletal muscle (14), leading to the decrease in insulin secretion in these mice.

The capillary area stained with lectin was significantly smaller in the islets from the ETIrs2KO mice

The capillary area stained with lectin was significantly smaller in the islets from the ETIrs2KO mice, compared with those from the control mice at 12 weeks (Fig. 5*A*, *B*). However, the number of capillaries in the islets was comparable between the control and the ETIrs2KO mice (Fig. 5*C*). Moreover, to assess islet oxygenation, we performed immunohistchemical staining for pimonidazole. No difference in the frequency of pimonidazole-positive islets was observed between the control and the ETIrs2KO mice (Fig. 5*D*). These findings suggest that the capillary area, but not the number of capillaries in the islets or islet oxygenation is reduced in the ETIrs2KO mice.

The islet blood flow was improved by treatment with an ACE-inhibitor, enalapril maleate, resulting in the amelioration of insulin secretion in the ETIrs2KO mice

Recent studies have demonstrated that prevention of angiotensin II-formation, by ACE inhibition with enalapril maleate, could preferentially increase the islet blood flow as a result of a vasodilating action (21). It had been demonstrated that islet microvessels may produce higher levels of angiotensin II than microvessels in the exocrine pancreas, and therefore may be more sensitive to ACE-inhibition in islets. In this study, the administration of enalapril maleate had no effect on the mean blood pressure (Fig. 6*A*), blood glucose concentrations (Table 2), or pancreatic blood flow (Fig. 6*B*), as reported

previously (21). However, the islet blood flow in the ETIrs2KO mice was restored significantly, almost to a level comparable to that in the control mice at 9-12 weeks (Fig. 6*C*). Furthermore, although enalapril maleate had no effect on the glucose-induced insulin secretion from isolated islets (Fig. 6*D*), the glucose-induced insulin secretion and glucose intolerance in the ETIrs2KO mice were significantly restored to levels equal to those in the control mice (Fig. 6*E*). These results suggest that the impairment of insulin secretion might have been caused by the impaired islet blood flow.

DISCUSSION

In this study, we demonstrated that the absence of Irs2 in endothelial cells impairs insulin secretion (Fig. 1*A-D*) by reducing the islet blood flow (Fig. 4*D*). In fact, following treatment with enalapril maleate, the glucose-stimulated insulin secretion was restored to levels equal to those observed in the control mice (Fig. 6*E*), along with an improvement in the islet blood flow (Fig. 6*C*). These data suggest that Irs2 in endothelial cells regulates islet blood flow, mediating insulin secretion. Although several studies have suggested that impaired insulin secretion was predominantly caused by β-cell dysfunction (22, 23), the absence of Irs2 in the capillaries also might cause insulin secretion in type 2 diabetes.

A recent study revealed that the correct integrity of the islet microvasculature is essential for normal islet function, as it not only provides the means for the transport of nutrients and oxygen but also ensures adequate paracrine interactions within the individual islets. β-cell-specific vascular endothelial growth factor-A gene ablation resulted in glucose intolerance and diabetes (24). These mice exhibited a decreased density of the microvasculature, and the capillaries exhibited an abnormal

morphological appearance. Moreover, β-cell-specific FRK tyrosine kinase transgenic mice exhibited an impaired glucose-stimulated insulin secretion in vivo (25). Insulin secretion in isolated islets from these mice was similar to that of control mice. However, the islet blood flow and capillary lumen diameter in the islets were decreased in these mice. In these genetically modified animals, although insulin secretion from isolated islets was maintained, insulin secretion was significantly impaired in vivo. These data suggest that the disorganization of islet vascularization can impair glucose-stimulated insulin secretion, even if the function of β-cells is not impaired. Moreover, recent investigations have demonstrated that pancreatic islet adaptation to insulin resistance is not limited to change within β-cells but also involves islet-specific neurovascular remodeling. To accommodate the increased demand for insulin delivery into the peripheral circulation, islet capillaries expand by dilation and not by angiogenesis (26). Given these findings, both the correct integrity and function of the capillaries in the islets might be essential for normal insulin secretion.

Why was the pancreatic blood flow similar but significantly decreased in the islets from ETIrs2KO mice? In a previous study, although the capillary blood flow after insulin treatment was impaired in the skeletal muscle of ETIrs2KO mice, this phenomenon was not observed during fasting (14). These data suggest that endothelial insulin signaling regulates capillary blood flow after insulin stimulation, but not in the basal state. It is possible that the islet blood flow in the ETIrs2KO mice was decreased because of the fact that the capillaries of the islets are constantly exposed to a high concentration of insulin, whereas the pancreatic blood flow is not because of the lack of insulin stimulation. In fact, the capillary blood flow was significantly higher in the islets than in the whole pancreas in the control mice (Fig. 4*B*, *D*, 1.38 ± 0.16 vs. 0.73 ± 0.07,

$P < 0.01$).

In a present study, the islet blood flow was significantly decreased in the ETIrs2KO mice. Although precise mechanisms remain unclear, it seems more likely that it was due to microvascular dysfunction, but not anatomical abnormalities. In fact, following treatment with enalapril maleate, the islet blood flow was restored to levels equal to those observed in control mice via vasodilating action (Fig. 6*C*). In addition, we did not detect anatomical abnormalities in islets of ETIrs2KO mice under the vascular staining (Fig. 5*A*, *C*) or the electron microscope (date not shown). Further study is needed to address this issue.

Recent trials have suggested that inhibitors of RAS, such as ACE inhibitors and ARBs, may reduce the incidence of new-onset type 2 diabetes in patients with or without hypertension who have a higher risk of developing diabetes (27-29). In addition to the amelioration of insulin secretion through the increased islet blood flow induced by an ACE inhibitor, as seen in this study, the blockade of the RAS during the development of diabetes has been attributed to improvements in peripheral insulin sensitivity and β-cell dysfunction (30, 31). ACE inhibitors have been shown to improve whole-body and skeletal muscle insulin resistance in hypertensive subjects with or without type 2 diabetes (32-40). The reduction of angiotensin II-mediated vascular resistance by ACE inhibition may improve insulin-stimulated glucose transport activity in skeletal muscle (41). This improvement is associated with a favorable adaptive response in glucose transporter-4 protein levels, glycogen storage, and the activities of relevant intracellular enzymes of glucose catabolism (42). Moreover, locally generated and physiologically active RAS components have functions that are distinct from the classical vasoconstriction and fluid homeostasis actions of systemic RAS. Local RAS

can affect islet-cell function and structure in the adult pancreas as well as the proliferation and differentiation of pancreatic stem/progenitor cells during development (43, 44). In fact, the blockade of the RAS significantly attenuates islet damage and restores the β-cell mass by reducing oxidative stress, apoptosis, and attenuating profibrotic pathways (42, 45). Thus, RAS inhibition may decrease the development and progression of diabetes through hemodynamic and non-hemodynamic effects or through the protection of β-cells and non-β-cells.

In conclusion, we demonstrated that the absence of Irs2 in endothelial cells impairs the islet blood flow, which may be one of the mechanisms responsible for the decrease in insulin secretion. Thus, Irs2 in endothelial cells may serve as a novel therapeutic target for preventing and ameliorating type 2 diabetes and metabolic syndrome.

Author Contributions. S.H., N.K., H.S., K.U. and T.K. designed this study and wrote the manuscript. S.H., N.K., H.S., M.S., I.T., T.K. and K.N. conducted the experimental research and analyzed the data. M.N. contributed to the data analysis and the preparation of the manuscript. T.K. is the guarantor of this work, and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

Acknowledgments. We wish to express our sincere appreciation to Dr. Wakako Fujimoto and Dr. Susumu Seino (Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan) for their advice and assistance in the development of our perfusion experiments. In addition, we thank Emi Hashimoto-Koga, Katsuko Takasawa, Norie Kowatari-Ohtsuka, Eri Yoshida-Nagata, Ritsuko Hoshino, Eishin Hirata, Namiko Okajima-Kasuga and Hiroshi Chiyonobu for

their excellent technical assistance and animal care.

Funding. This work was supported by a grant for TSBMI from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Grant-in-aid for Scientific Research in Priority Areas (A) (18209033) and (S) (20229008) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. K.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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Table 1. Effect of insulin on blood glucose level.

Strain	Control	ETIrs2KO
No. of animals	10	10
The change of blood glucose, mmol/L	-2.69 ± 0.35	-2.58 ± 0.47

Values are the mean ± SE

Table 2. Effect of enalapril maleate on blood glucose and plasma insulin levels.

Strain	Control	ETIrs2KO	ETIrs2KO
Substance given	Saline	Saline	Enalapril maleate
No. of animals	10	10	10
Blood glucose, mmol/L	6.23 ± 0.19	6.47 ± 0.20	6.21 ± 0.24
Plasma insulin, nmol/L	0.23 ± 0.03	0.26 ± 0.05	0.22 ± 0.04

Values are the mean ± SE

FIGURE LEGENDS

Figure 1- Insulin secretion was impaired in the ETIrs2KO mice. Blood glucose levels (left) and plasma insulin levels (right) during an intraperitoneal glucose tolerance test in the control and the ETIrs2KO mice at 12 (*A*) and 24 (*B*) weeks (n = 9-10). (*C*) Plasma insulin levels during an intraperitoneal glucagon tolerance test in the control and the ETIrs2KO mice at 14 weeks (n = 9-10). (*D*) Plasma insulin levels during an intraperitoneal arginine tolerance test in the control and the ETIrs2KO mice at 13 weeks (n = 9-10). Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$).

Figure 2- Insulin secretion in the isolated islets was not impaired in the ETIrs2KO mice. (*A*) Glucose-induced insulin secretion using batch-incubated islets at 12 weeks (n = 9). (*B*) Glucose-induced insulin secretion as assessed in an islet perifusion experiment at 12 weeks (n = 8). (*C*) mRNA expression levels expressed as the values in the islets of the ETIrs2KO mice relative to those of the control mice at 12 weeks (n = 5). (*D*) Insulin content per DNA concentration in islets isolated from the control and the ETIrs2KO mice at 12 weeks (n = 8). (*E*) Insulin staining of pancreatic sections from the control and the ETIrs2KO mice at 24 weeks (scale bar = 100 μm). (*F*) The β-cell mass was calculated as estimated islet weight (n = 5). (*G*) Replication rate of β-cells assayed on the basis of BrdU incorporation in the control and the ETIrs2KO mice at 24 weeks (n = 7). Results are shown as the percentage of BrdU-positive cells relative to the total number of β-cells. Values are the mean ± SE.

Figure 3- Insulin secretion was significantly decreased in the ETIrs2KO mice during pancreatic perfusion. (*A*) Insulin secretion from perfused pancreata of the control and

the ETIrs2KO mice at 12 weeks (n = 8). (*B*) Amounts of insulin secreted in the control and the ETIrs2KO mice after glucose stimulation expressed as the $AUC_{insulin}$ values from 5 to 30 min in *A*. Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$) and ** ($P < 0.01$).

Figure 4- The islet blood flow was significantly decreased in the ETIrs2KO mice. The mean blood pressure (*A*), pancreatic blood flow (*B*), the number of microspheres in islet tissue (*C*) and islet blood flow (*D*) in anaesthetized 9-12-week-old control and ETIrs2KO mice in the basal state (n = 10). The islet blood flow (*E*) in anaesthetized 9-week-old control and ETIrs2KO mice in insulin-treated state (n = 10) Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$) and *** ($P < 0.001$).

Figure 5- The capillary area stained by lectin was significantly decreased in the islets of the ETIrs2KO mice. Lectin staining of pancreatic section from the control and the ETIrs2KO mice at 12 weeks (scale bar = 50 μm). The approximate islet boundary is marked by a dotted line (*A*). Vessel area expressed as a relative value to the total islet volume % (*B*) and the number of capillaries per square millimeters of islet (*C*). Pimonidazole-positive islets were quantitatively assessed as a percentage of the total number of islets (*D*). Values are the mean ± SE for 5 animals. Statistical significance is depicted as *** ($P < 0.001$).

Figure 6- The islet blood flow was improved by treatment with an ACE-inhibitor, enalapril maleate, resulting in the amelioration of insulin secretion in the ETIrs2KO mice. The mean blood pressure (*A*), the pancreatic blood flow (*B*), and the islet blood

flow (*C*) in anaesthetized 9-12-week-old control and ETIrs2KO mice at 10 min after the intravenous injection of saline or enalapril maleate (n = 10). (*D*) Effect of enalapril maleate on glucose-induced insulin secretion using batch-incubated islets of C57BL/6J mice at 12 weeks (n = 5). (*E*) Blood glucose levels (left) and plasma insulin levels (right) during an intraperitoneal glucose tolerance test in 12-week-old control and ETIrs2KO mice at 10 min after the intravenous injection of saline or enalapril maleate (n = 11-13). Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$, control + saline vs. ETIrs2KO mice + saline) and # ($P < 0.05$, ETIrs2KO mice + saline vs. ETIrs2KO mice + enalapril).

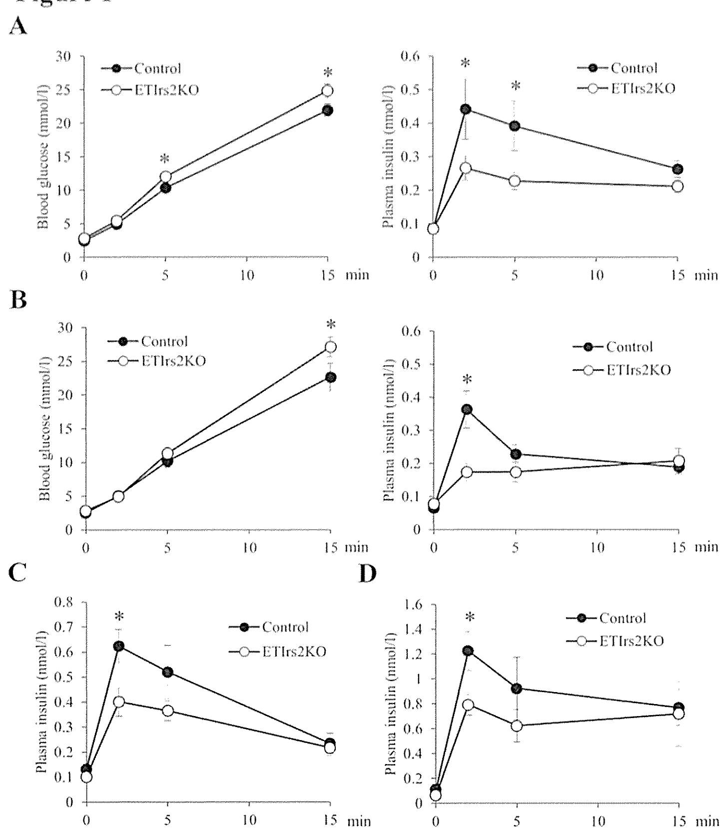

Figure 1- Insulin secretion was impaired in the ETIrs2KO mice. Blood glucose levels (left) and plasma insulin levels (right) during an intraperitoneal glucose tolerance test in the control and the ETIrs2KO mice at 12 (A) and 24 (B) weeks (n = 9-10). (C) Plasma insulin levels during an intraperitoneal glucagon tolerance test in the control and the ETIrs2KO mice at 14 weeks (n = 9-10). (D) Plasma insulin levels during an intraperitoneal arginine tolerance test in the control and the ETIrs2KO mice at 13 weeks (n = 9-10). Values are the mean ± SE. Statistical significance is depicted as * ($P < 0.05$).
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