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Regular Article

Protective Effect of Ebselen on Ischemia-reperfusion Injury in Epigastric Skin Flaps in Rats

Takahiko Kuroki[1], Susumu Takekoshi[2], Kanae Kitatani[3], Chikara Kato[2], Muneo Miyasaka[1] and Tadashi Akamatsu[1]

[1]Department of Plastic Surgery, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan, [2]Department of Cell Biology, Division of Host Defense Mechanism, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan and [3]Support Center of Medical Research and Education, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan

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The purpose of this study was to determine the role of oxidized diacylglycerol (DAG) and the molecular mechanism underlying ischemia-reperfusion (I/R) injury in rat skin flaps. The protective effect of ebselen on the viability of rat skin flaps with I/R injury was investigated. Flaps were designed and raised in the left inguinal region. Then, a microvascular clamp was applied to the vascular pedicle and reperfused after 6 hr. After 7 days of I/R (I/R group), the skin flap survival area ratio was significantly reduced compared to the normal skin. The administration of ebselen significantly improved the ratio compared to the I/R group. The flap survival area ratio of the I/R + ebselen group was significantly improved compared to the I/R + vehicle group. In the I/R + ebselen group, the oxidized DAG content and intensity of phosphorylated PKCα and PKCδ were significantly lower compared to the I/R + vehicle group. Furthermore, the inflammatory response was suppressed in the I/R + ebselen group compared to the I/R + vehicle group. These results indicate that ebselen is useful as a preventive and therapeutic agent for skin flap necrosis caused by I/R, because of reduction and elimination of oxidized DAG.

Key words: skin flap, ischemia-reperfusion injury, oxidized diacylglycerol, protein kinase C, ebselen

I. Introduction

Reconstructive surgery for tissue loss due to trauma or cancer surgery significantly improves the return to society and quality of life of patients. However, reconstructive surgery has certain complications, including necrosis of the transplanted tissue. Skin flap surgery can cause embolization in the vasculature in 10–15% of cases and complete or partial necrosis of the skin flap in 0.9–5.6% of cases [8, 11]. It is crucial to prevent skin flap necrosis and improve its survival. Necrosis of the skin flaps can be caused by two factors: impaired blood flow (such as insufficient arterial blood flow or venous stasis) and reperfusion injury. Various organ tissues exposed to ischemia undergo oxidative stress triggered by the influx of oxygen molecules during reperfusion, resulting in more severe tissue injury [15, 19, 20, 28, 34]. Many studies have evaluated the relationship between oxidative stress due to I/R and flap necrosis; however, the process leading to flap tissue injury and its underlying molecular mechanism have not been clarified [6, 23, 41].

Reactive oxygen species (ROS) generated by I/R cause cell injury through the oxidation of biomolecules [18, 38, 40]. Polyunsaturated fatty acids, which have multiple double bonds, are particularly susceptible to oxidation and lipid peroxidation plays a central role in oxidative stress injury. Protein kinase C (PKC) is activated by diacylglyc-

Correspondence to: Susumu Takekoshi, Department of Cell Biology, Division of Host Defense Mechanism, Tokai University School of Medicine 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan.
E-mail: takekos@is.icc.u-tokai.ac.jp

erol (DAG), a lipophilic signaling molecule, and plays an important role in various biological processes, including cell proliferation, development, differentiation, and apoptosis [1, 21, 22, 43]. Takekoshi *et al.* demonstrated that oxidized DAG can more strongly activate PKC than unoxidized DAG [35]. Toriumi *et al.* also reported that oxidized DAG is generated in carbon tetrachloride (CCl_4)-induced liver injury, resulting in activation of the PKC/NF-κB pathway and upregulation of TNFα-mediated aggravation of inflammation [37]. Haraguchi *et al.* showed that in diabetic nephropathy, oxidative stress causes cellular defects in glomeruli and proximal renal tubules through the NF-κB signaling pathway [12]. Furthermore, Takekoshi *et al.* showed that oxidized DAG-induced PKC activation, phosphorylation of ERK and JNK, and increase in TIMP-1 and TNF-α in mice liver with hepatic fibrosis were induced by long-term administration of CCl_4 [36]. In addition, activated PKC exacerbates diseases associated with oxidative stress, such as chronic liver disease, hematological malignancies, and cerebrovascular disorders [5, 7, 25]. These findings suggest that oxidized DAG is a key molecule in diseases caused by oxidative stress. Although oxidized DAG is involved in I/R injury in skin flaps, the details are unknown.

Antioxidant enzyme systems protect against oxidative stress injury. One of the well-known antioxidant enzymes is glutathione peroxidase (GPx). GPx contains selenium in its active site, which reduces lipid peroxides in the presence of glutathione. Various organs have disrupted balance between the ROS generating and scavenging systems. In particular, arteriosclerosis and male infertility are caused by decreased GPx [14]. Kambayashi *et al.* showed that GPx4, which reduces phosphatidylcholine hydroperoxide, reduces oxidized DAG [16]. Ebselen, which was developed for the treatment of acute stroke, is a lipid-soluble seleno-organic compound that effectively reduces phospholipid hydroperoxides through GPx4-like activity in the presence of glutathione (Fig. 1) [26]. This suggests that ebselen reduces oxidized DAG. In addition, ebselen has numerous anti-inflammatory effects in animal models of I/R injury in the brain, testis, and intestinal tract, as well as after lung transplantation [9, 10, 30, 31]. In this study, it was considered that ebselen reduces oxidized DAG and suppresses I/R injury of the skin flap. The purpose of this study was to elucidate the role of oxidized DAG in I/R injury using a rat skin flap model. In addition, the effects of ebselen administration on the scavenging of oxidized DAG and protection of skin flaps were investigated.

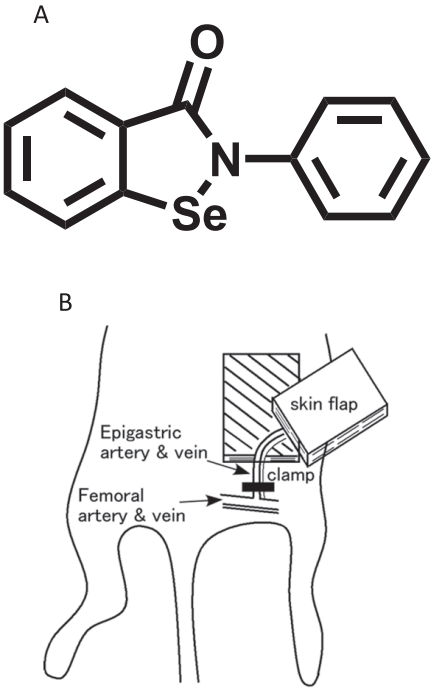


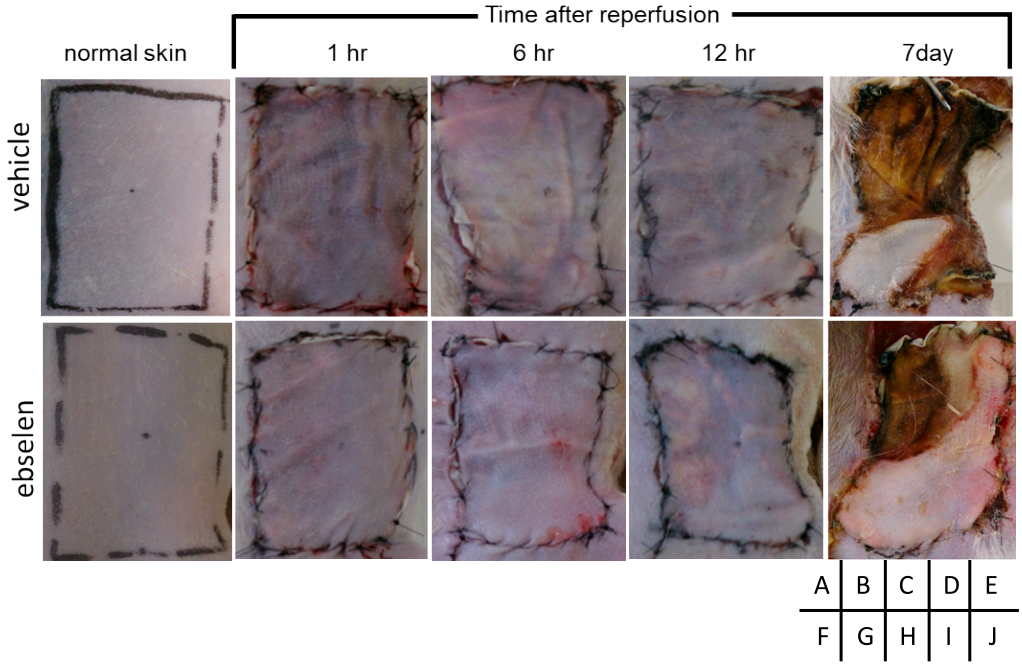
Fig. 1. Chemical structure of ebselen and experimental schematic diagram of I/R. **A**: Chemical structure of ebselen. **B**: Experimental schematic diagram of I/R injury skin flap model. Skin flap with a superficial epigastric vessel as the pedicle was elevated in the left groin.

II. Materials and Methods

Experimental model

All animal experiments were approved by the Animal Experimentation Committee, Isehara campus (Tokai University, Kanagawa, Japan). Sixty male Sprague-Dawley rats (8 weeks of age, 270–330 g, Clare Japan, Tokyo) were used. Rats were anesthetized with 2.5% isoflurane and air. The left groin was shaved and a 45 × 30-mm pedicled skin flap with a superficial epigastric vessel as the pedicle was elevated in the left groin (Fig. 1) [27]. Then, the skin flap pedicles were clamped and the elevated skin flap was sutured as before with the interposition of a rubber sheet at the lower surface of the flap to prevent neovascularization from the wound bed. After the surgery, a cast was applied to the abdomen to prevent auto-mutilation. After ischemia for 6 hr, the blood flow was resumed by removing the clip under anesthesia. The skin flap was collected at postoperative 1, 6, and 12 hr, and 7 days. Six rats were included in each group. Since no necrosis occurred in normal skin and in skin at 1, 6, and 12 hr after I/R, biochemical and histochemical analyses were performed on the central portion of the skin flap that was considered most likely to have experienced tissue injury. Seven days after I/R, we evaluated the most cephalic central portion to determine the changes in the area closest to the site of tissue injury [4].

A portion of the collected tissue was snap-frozen in liquid nitrogen and stored at −80°C, for biochemical analysis. The remaining tissue was used to create a paraffin-embedded block and frozen block. A portion of the tissue was fixed by overnight immersion in Mildform 10N (Wako, Osaka, Japan), and a paraffin-embedded block was created according to the conventional method. To create the frozen block, the tissue was fixed by overnight immersion in 4%



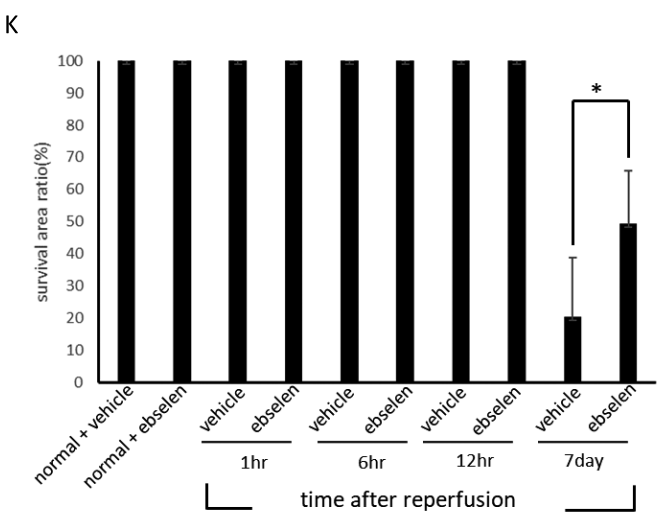


Fig. 2. Survival area of the skin flaps of vehicle administration and ebselen administration groups. (**A–E**) Vehicle (distilled water) administration group. (**A**) Normal skin. (**B**) 1 hr after I/R. (**C**) 6 hr after I/R. (**D**) 12 hr after I/R. (**E**) 7 days after I/R. (**F–J**) Ebselen administration group. (**F**) Normal skin. (**G**) 1 hr after I/R. (**H**) 6 hr after I/R. (**I**) 12 hr after I/R. (**J**) 7 days after I/R. (**K**) Comparison of survival area ratio in each group. Each bar represents mean ± SD for six rats. *$p < 0.05$.

PFA/PB, replaced with sucrose/PBS solution, and embedded in OCT compound. In the I/R + ebselen group, ebselen (100 mg/kg) was administered orally using a gastric tube, suspended in 500 µl of distilled water, immediately before the surgery, 1 hr before reperfusion, 6 hr after reperfusion, and every 12 hr thereafter. In the I/R + vehicle group, rats received the same quantity of distilled water only and were considered as controls.

Survival area of the flap

To evaluate the skin flap injury, the area ratio of skin flap survival and necrotic regions was measured [24]. After I/R, areas of viable and non-viable flap were traced on a tracing paper; these paper templates were cut and weighed. The survival area ratio was calculated as the ratio of the weight of the survival area to that of the entire area. In addition, the degree of injury to the skin flap in each group was compared [39].

Assay of oxidized DAG

For the assay of oxidized DAG, the lipids were extracted from frozen skin flap tissues using 2-propanol containing 1-palmitoyl-3-arachidoylglycerol hydroxide (as an internal standard), 20 mM butylated hydroxytoluene (Wako, Osaka, Japan), and 200 mM triphenylphosphine (Wako, Osaka, Japan). Butylated hydroxytoluene and triphenylphosphine were added to prevent artefactual oxidation during the analytical procedure and reduce hydroperoxide to hydroxide.

The lipids extracted using reverse-phase high-performance liquid chromatography (HPLC) were eluted fractions of oxidized DAG. The collected fraction was separated by a forward-phase HPLC to collect the elution fraction of the oxidized DAG. Then, the oxidized DAG was labeled with pyrene-1-carbonyl cyanide in the presence of 5 mg/ml of quinuclidine. The labeled oxidized DAG was detected by reverse-phase HPLC coupled with fluorescence detection, and the amount per tissue weight of DAG was measured.

Immunohistochemistry

Frozen sections were used for immunohistochemical analysis of phosphorylated PKCα and δ. This was because (1) frozen sections retain antigenicity better than paraffin sections and (2) the manufacturer of the antibodies recommends the use of frozen sections for immunohistochemical analysis of phosphorylated PKC. The frozen block was sliced into 10-μm sections. The sections were reacted with the primary antibody overnight at 4°C, after endogenous peroxidase treatment with 99 ml of methanol and 1 ml of 30% hydrogen peroxide. The primary antibodies used were anti-phosphorylated PKCα antibody (1:100; Cell Signaling Technology), anti-phosphorylated PKCδ antibody (1:100; Cell Signaling Technology), and anti-myeloperoxidase antibody (MPO) (1:500; Meridian Life Science), diluted in 1% BSA/PBS. After washing the primary antibody, the secondary antibody was reacted and visualized using 3,3'-diaminobenzidine. PKC was activated by migration from the cytoplasm to the cell membrane and binding to the DAG. Activated PKC molecules were self-phosphorylated. Therefore, PKC activation was an index marker of PKC phosphorylation.

Immunohistochemistry of native PKCα and PKCδ was performed using paraffin-embedded blocks. The 6-μm paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded series of ethanol. Then, the sections were reacted with the primary antibodies, rabbit IgG against PKCα, PKCδ (1:100; Santa Cruz) in 1% BSA/PBS overnight at 4°C. After washing the primary antibodies, the secondary antibody was added and the sections were visualized with 3,3'-diaminobenzidine. The immunohistochemical staining intensity was rated on a 4-point scale: 0 (very weak), 1 (weak), 2 (strong), and 3 (very strong). The evaluation was blindly performed by two researchers, with six samples per group.

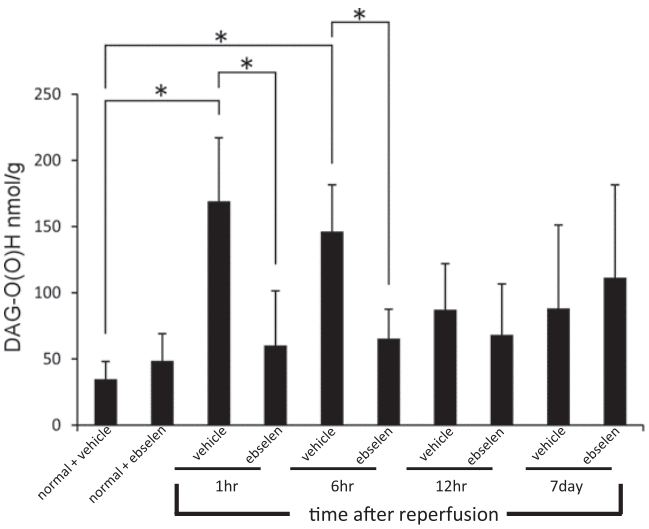


Fig. 3. Quantitative analysis of oxidized DAG. Pyrene-fluorescence labeling techniques were used to investigate the formation of oxidized DAG in the skin flaps at 1, 6, and 12 hr after I/R in the ebselen administration and vehicle administration groups. Each bar represents mean ± SD for six rats. *$p < 0.05$.

Histological examination

Tissue samples were stained with hematoxylin and eosin (H&E), and the flap condition was observed.

Statistical analysis

Values are expressed as mean ± SD. Differences were analyzed using Student's *t*-test, and statistical significance was set at $p < 0.05$.

III. Results

I/R improved the tissue injury

Skin flap injury after I/R was evaluated by calculating the survival area ratio on day 7 after reperfusion (Fig. 2). The survival area ratio of the I/R + vehicle group was 20.4%, thereby showing marked tissue injury compared to the normal skin. In contrast, the flap survival area ratio of the I/R + ebselen group was 49.3%, thereby showing significantly improved tissue injury.

Quantitative determination of oxidized DAG

The oxidized DAG was quantified by HPLC using a fluorescence detector (Fig. 3). In the I/R + vehicle group, oxidized DAG showed a significant increase compared to the normal skin at 1 and 6 hr after I/R. In addition, oxidized DAG was significantly reduced in the I/R + ebselen group compared to the I/R + vehicle group at 1 and 6 hr after I/R.

Phosphorylation of PKCα and δ in the skin flaps

Using phosphorylation as an indicator, activation of PKC molecules was observed. Phosphorylated PKCα was strongly stained in the dermis and epidermis compared to the normal skin in both groups at 1, 6, and 12 hr after I/R.

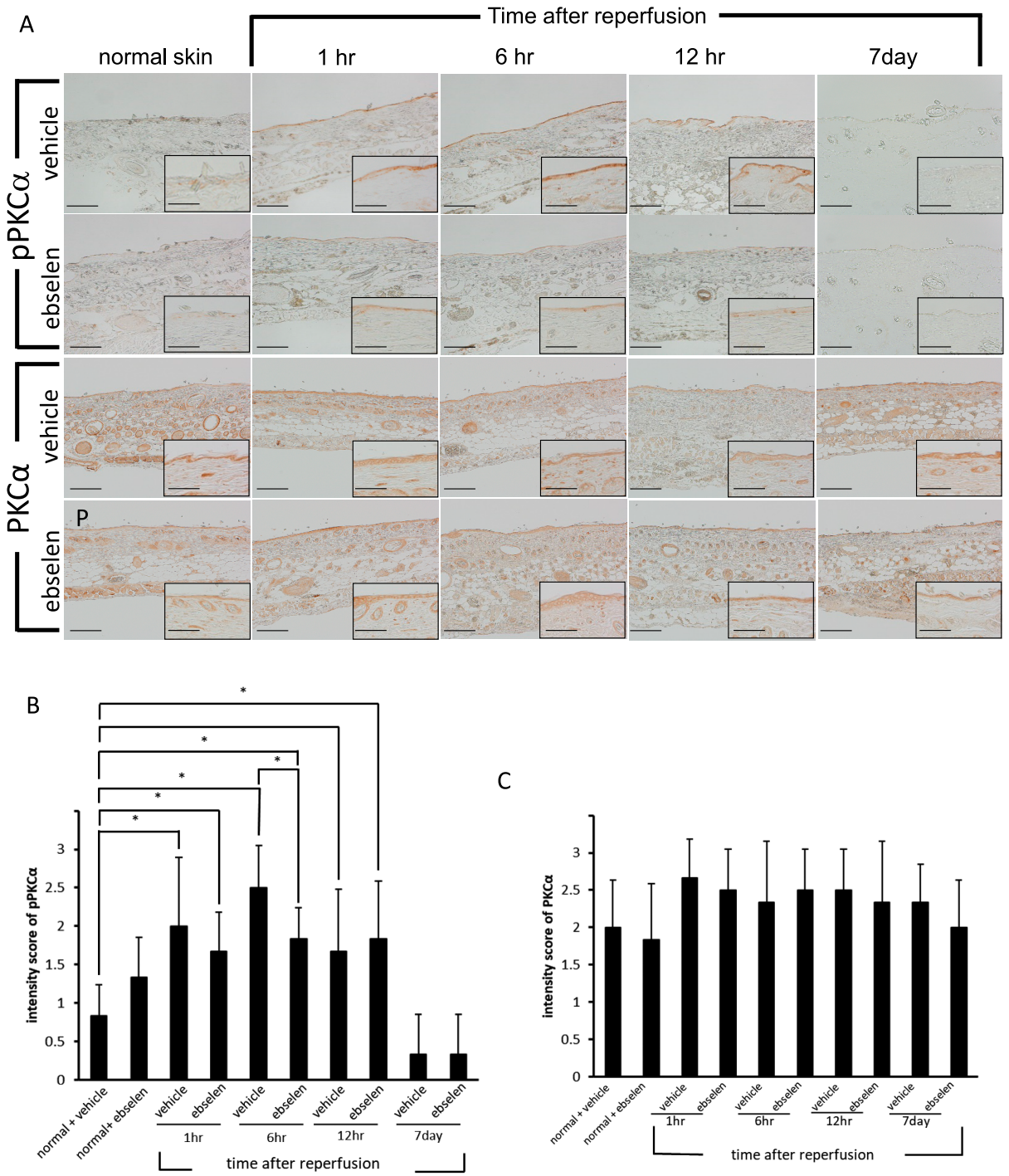


Fig. 4. Immunohistochemical analysis for phosphorylated PKCα and PKCα in I/R-induced skin flap injury. (**A**) Skin flap from vehicle administration and ebselen administration groups harvested at 1 hr, 6 hr, 12 hr, and 7 days after I/R were stained with anti-PKCα antibodies and anti-phosphorylated PKCα antibodies. Representative sections are presented (bar = 100 μm). Inserts are enlarged views (bar = 200 μm). (**B**) Comparison of intensity score for phosphorylated PKCα in each group. (**C**) Comparison of intensity score for PKCα in each group. Each bar represents mean ± SD for six rats. *$p < 0.05$.

This suggested that PKCα activation was caused by the increase in oxidized DAG in the skin flap tissue. In addition, strong immunostaining for phosphorylated PKCα was observed in the I/R + vehicle group, whereas the staining intensity was suppressed in the ebselen-treated group. Immunostaining for non-phosphorylated PKC α showed no difference in staining intensity between the groups (Fig. 4). The immuno-stainability of phosphorylated PKCδ was also increased at 1, 6, and 12 hr after I/R groups compared to the normal skin, indicating increased PKCδ activation.

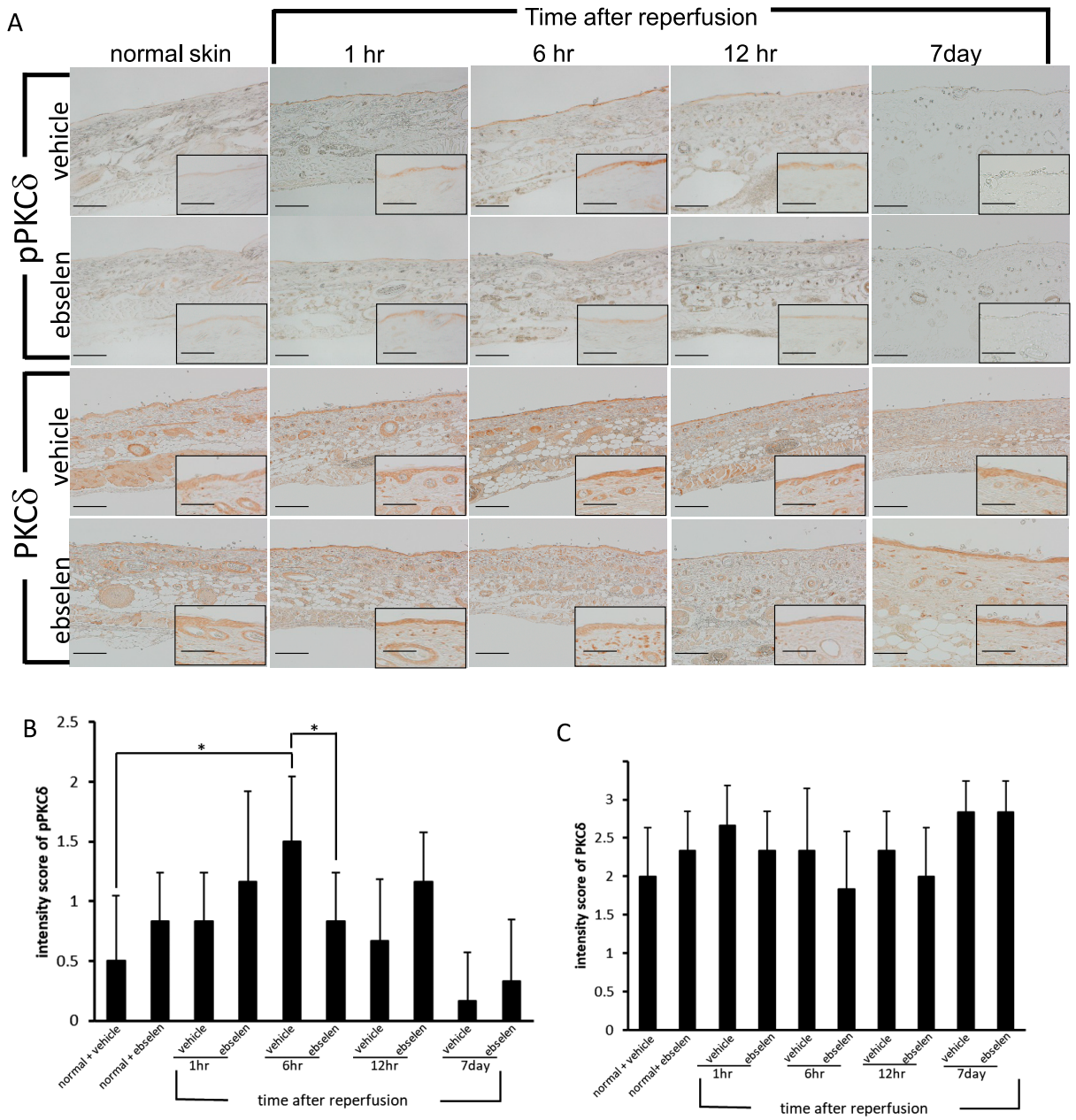


Fig. 5. Immunohistochemical analysis for phosphorylated PKCδ and PKCδ in I/R-induced skin flap injury. (**A**) Skin flap from vehicle administration and ebselen administration groups harvested at 1 hr, 6 hr, 12 hr, and 7 days after I/R were stained with anti-PKCδ antibodies and anti-phosphorylated PKCδ antibodies. Representative sections are presented (bar = 100 μm). Inserts are enlarged views (bar = 200 μm). (**B**) Comparison of intensity score for phosphorylated PKCδ in each group. (**C**) Comparison of intensity score for PKCδ in each group. Each bar represents mean ± SD for six rats. *$p < 0.05$.

After 6 hr of I/R, the staining intensity was significantly reduced in the I/R + Ebselen group. Immunostaining for the non-phosphorylated PKC δ showed no difference in staining intensity between the groups (Fig. 5). In the I/R groups, PKCα and PKCδ were activated. It has been reported that over-activation of PKCα and PKCδ leads to exacerbation of NFκB-mediated inflammation, resulting in tissue injury [3, 17, 33, 37, 45].

Neutrophil invasion of the skin flaps

To investigate tissue injury and inflammatory cell infiltration, we performed H&E staining and immunohistochemistry of MPO, a protein marker of neutrophils at 7 days after I/R (Fig. 6). The results showed marked neutrophil infiltration into the tissue from the deep dermis in the I/R + vehicle group, and suppressed neutrophil infiltration in the I/R + ebselen group.

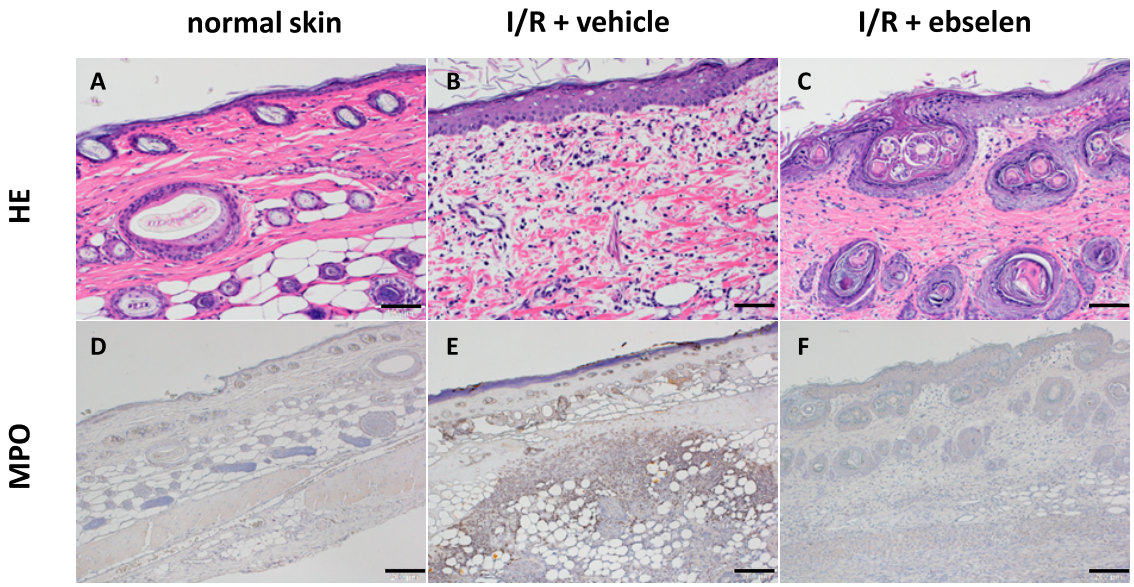


Fig. 6. Neutrophilic infiltration at 7 days after I/R. (**A–C**) Hematoxylin and eosin stain. (**A**) Normal skin in the vehicle administration group. (**B**) I/R + vehicle administration group. (**C**) I/R + ebselen administration group. (**D–F**) Myeloperoxidase stain. (**D**) Normal skin in the vehicle administration group. (**E**) I/R + vehicle administration group. (**F**) I/R + ebselen administration group. Bar = 200 μm.

IV. Discussion

Our results showed increased oxidized DAG after I-R, which induced overactivation of PKCα and PKCδ, resulting in inflammation in the skin flap. However, at certain time points, the increase in the quantity of oxidized DAG did not coincide with increased phosphorylation of the PKC molecules. Oxidized DAG was significantly increased at 1 and 6 hr after I/R. On the other hand, phosphorylated PKCα was significantly increased at 1, 6, and 12 hr after I/R. The quantity of oxidized DAG at 12 hr after I/R was increased, although without statistical significance. The increased oxidized DAG may have caused increased phosphorylation of PKCα at 12 hr after I/R. PKCδ phosphorylation was significantly increased only at 6 hr, and showed an increasing trend at 1 and 12 hr, although the increase was not significant. This increase corresponded to an increase in oxidized DAG. These results suggest that at 1, 6, and 12 hr after I/R, oxidized DAG was increased, thereby increasing the levels of phosphorylated PKCα and PKCδ. The increase in oxidized DAG and PKC activation that occurs early after I/R (1–12 hr) may have triggered the skin injury at 7 days after I/R.

Furthermore, pre-administration of ebselen prior to reperfusion significantly suppressed phosphorylation of PKCα and PKCδ by inhibiting the production of oxidized DAG after reperfusion in early phase, thereby improving the survival area ratio of skin flap tissue at 7 days after I/R. These findings demonstrated for the first time that ebselen may be used for prophylaxis against the necrosis of skin flap caused by I/R injury. Kambayashi *et al.* reported that oxidized DAG induces neutrophil activation via PKC activation in experiments with human neutrophils [16]. Toriumi *et al.* showed that in rat liver induced with oxidative stress, the increased oxidized DAG causes overactivation of PKCα, which exacerbates the inflammatory responses via NF-κB activation and increased production of TNF-α [37]. This suggests that PKC activation by the increase in oxidized DAG also induced neutrophil infiltration and tissue injury in the skin flap after I/R.

A number of studies have used animal models of skin flap I/R injury. Angel *et al.* reported that malondialdehyde, a metabolite of lipid peroxides, was elevated with ROS production in the skin flap after I/R injury [2]. Han *et al.* showed that TNF-α expression was induced in the skin flap after I/R injury via activation of NF-κB [11].

In addition, administration of antioxidants (such as vitamin C), iron chelator deferoxamine, superoxide dismutase, and allopurinol increases skin flap survival after I-R injury in rats [2, 13, 44]. Moreover, Personelle *et al.* reported that local injection of vitamins C and E, which are antioxidants, for 15 days after reconstructive surgery improved the necrotic skin flaps in humans [29]. Thus, oxidants, such as lipid peroxides, are involved in I/R injury of the skin flap, but the detailed molecular mechanism has not been clarified. Many drugs, such as antioxidants, reduce I/R injury of skin flaps, but have not been applied to clinical practice. In this study, it was shown for the first time that oxidized DAG, a lipid peroxide, increases in the early stages of I/R injury in skin flaps. In addition, ebselen treatment significantly reduced oxidized DAG and suppressed tissue damage, strongly suggesting that oxidized DAG is a key mediator of tissue damage in skin flaps. Ebselen is a low molecular weight antioxidant with the ability to reduce lipid peroxides. Ebselen inhibited I/R injury of skin flaps by reducing and scavenging oxidized DAG, and improved

the survival area ratio, suggesting its potential as a therapeutic agent for skin flaps. In Japan, ebselen has been shown to have therapeutic efficacy and safety in acute stroke patients in a Phase III clinical trial [42]. In addition, ebselen has been approved by the U.S. National Institutes of Health as safe for human use and recently been studied as a treatment for bipolar disorder [32]. It is expected that ebselen, which has already been shown to be safe, will be used as a therapeutic drug for skin flaps in the future.

In this study, we quantified oxidized DAG by HPLC and assessed PKC phosphorylation by immunohistochemistry. The activation of PKC was also verified by Western blotting using skin flap tissue, but phosphorylated PKC molecules, which are indicators of PKC activation, could not be detected (data not shown). It was speculated that this was due to the very small quantity of phosphorylated PKC in the whole skin flap tissue. Further verification using cultured epidermal tissues is necessary in the future.

In the present study, we clarified the role of oxidized DAG as a key molecule in I/R injury in skin flap. Furthermore, it was found that ebselen treatment could suppress the injury by reducing and eliminating oxidized DAG. At present, there is no information on the presence of oxidized DAG and PKC activation in human skin flap tissue. In the future, we plan to conduct a detailed study using human tissues for clinical application.

V. Conflicts of Interest

The authors have no conflict of interest to declare, in this study.

VI. Acknowledgments

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