Pro-angiogenic activity of isofuran in rat brain endothelial cells

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Abstract

Background: Isofurans (IsoFs) are a series of novel discovered lipid peroxidation products, which are found in some oxidation-injured disorders. Oxidative damage is a major pathological feature of abnormal vascularization diseases. This study is focused on the investigation of angiogenic property of IsoF. The alteration on cellular proliferation, migration, and apoptosis by an IsoF were also detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, coverslip migration assay, and TUNEL assay. Furthermore, cellular calcium release and extracellular regulated protein kinases (ERK) 1/2 activation by IsoF were analyzed. RT-PCR was carried out to detect some angiogenesis-related gene expression induced by IsoF.

Results: MTT stain assay indicated that 1mM IsoF had the most bioactivity in RBECs. IsoF significantly promoted cellular proliferation and migration and remarkably decreased staurosporine-induced apoptosis by TUNEL assay in the RBECs. Moreover, IsoF activated extracellular regulated protein kinases (ERK)1/2 and trigged calcium release. RT-PCR examination indicated that IsoF up-regulated tumor necrosis factor (TNF)a, angiopoietin-1 receptor (Tie2), and vascular endothelial growth factor (VEGF)-A, but did not interfere with caspase 2 and VEGF-C in the RBECs.

Conclusion: IsoF has pro-angiogenic activity *via* promotion of cellular proliferation/migration and prohibition of cellular apoptosis. Calcium release and ERK1/2 phosphorylation may be involved in the signaling of the IsoF-induced up-regulation of TNFa, Tie2, and VEGF-A, which could be the molecular mechanism of the pro-angiogenic activity of the IsoF.

Background

Cellular membrane is the major source of lipid which supplies the energy for cellular activities. Some lipid products can play a crucial role for cellular signaling to involve many important cellular processes. Isofurans (IsoFs) are series novel lipid peroxidative products, which are derived non-enzymatically from free radical mediated peroxidation of arachidonic acid. The structural feature of the IsoF is similar to another kind of lipid peroxidative product, isoprostaine (IsoP). Both of IsoF and IsoP are formed under similar conditions, however, IsoF is substituted with tetrahydrofuran ring at a relatively elevated oxygen environment [1]. It suggests that the oxidative stress environment is favored for IsoF formation. IsoF is detectable in normal fluid and tissue. Fessel *et al* found that the level of IsoF is increased dramatically in a CCl_4-induced oxidant injury rat model [1]. Furthermore, they revealed that the level of IsoF, but not IsoP, is significant higher in human substantia nigra than that of controls in some neurodegenerative diseases, Lewy body disease, and Parkinson's disease [2].

Angiogenesis is defined as a physiological process that novel blood vessels form from pre-existing vascular sprouts [3]. Physiological angiogenesis normally occurs in embryo and wound healing processes. However, dysregulation of blood vessel growth (excessive or insufficient angiogenesis) will cause a list of disorders [4]. Dysregulated vascularization is the major pathological character of oxidant

injury. A list of human discords have been reported to relate to lipid peroxidative damages in the conditions of which IsoF could be produced, such as atherosclerosis [5], cancer [6, 7], diabetes [8], chronic alcohol exposure [9], acute lung injury [10, 11], the neurodegenerative disorders [12], as well as angiogenesis [13] etc.

Based on these points, we speculate that IsoF may participate in angiogenic regulation. This study is focused on the angiogenic regulation potential analysis of the IsoF *in vitro* in rat brain endothelial cells (RBECs) and *in extra vivo* in rat aorta. We found that IsoF reveal a pro-angiogenic activity. Signaling pathways and angiogenesis-related genes, which may take part in the pro-angiogenic activity of the IsoF, are further investigated. These findings provide evidences to show the pro-angiogenic potential of IsoF. These preliminary data will open the study on the IsoF in lipid peroxidation- and angiogenesis-associated disorders.

Statistical analysis

Data are presented as means ± s.e.m. Comparisons between groups were made using one-way analysis of variance followed by post-hoc Tukey's multiple comparisons test among means. $p<0.05$ was considered statistically significant.

Results

Detection of the optimal concentration of pro-proliferation of IsoF

The angiogenic activity of IsoF was examined with cellular proliferation assay in the RBECs. Dose responses of the IsoF (from 1pM to 10mM) in cellular viability were detected by MTT assay (Fig. 1A). The maximal cellular viability responses were induced by IsoF at 1μM. Therefore, 1μM IsoF was selected for the next experiments.

Pro-angiogenic activity of IsoF in RBECs

Subsequently, the cellular proliferation and migration induced by 1μM IsoF were analyzed by MTT assay and Coverslip-removing migration assay in the RBECs. The results showed that IsoF, being similar to the 10% Growth Supplement medium, increased 1.8-folds of the cellular proliferation compared with that of the negative control (2% Growth Supplement medium) (Fig. 1B; $p<0.01$). Additionally, the cellular migration rate of the RBECs stimulated by 1mM IsoF was 16-folds higher than that of the negative cells (Fig. 1C; $p<0.01$). These data clearly suggested that the IsoF has a pro-angiogenic potential.

Cellular protective activity of IsoF in RBECs

Cellular protective activity of the IsoF was then analyzed by TUNEL stain in the RBECs. Comparing to the staurosporine-induced apoptotic RBECs (PBS-pre-treatment, 75% apoptotic rate), pre-treatment with 1mM IsoF significantly prevented the staurosporine-induced cellular apoptosis (29% apoptotic rate) (Fig. 2; $p<0.05$). It suggested that IsoF have a cellular protective potential in the RBECs.

IsoF induced cellular calcium release in RBECs

The chemical structure future of the IsoF is short lipid peroxidative products with multi-alkene and multi-alkane structures [1, 14]. Interestingly, multiple similar structure lipid products are the ligands of G protein-coupled receptors (GPCRs or GPRs). For instance, natural cannabinoids (Cannabigerol-type), linoleic acid, abnormal-cannabidiol, and free fatty acids are specific ligands of cannabinoids receptor 1 (CB1R) and CB2R[15, 16], GPR40 [17], GPR55 [18], and GPR120 [19], respectively. Therefore, we further investigated the typical GPCR signal pathways, calcium release and extracellular regulated protein kinases (ERK)1/2 activation. Comparing to the PBS stimulation, 1mM IsoF quickly and clearly triggered cellular calcium release in RBECs in a Fluo-4 NW fluorescence labeling assay system (Fig. 3).

IsoF activated ERK1/2

EKR1/2 activation induced by the IsoF was then detected as well in a time-course using an immune blot assay in the RBECs. Notably, the ERK1/2 activation (phosphorylation) in the RBECs was observed after a 2-minute stimulation with 1μM IsoF and reached the maximal activation level at 20-minute stimulation by the IsoF (Fig. 4). The data above seems to suggest that the signaling of the angiogenic activity of the IsoF may pass through both the calcium release and the ERK1/2 activation.

Angiogenic and cell protective factor gene regulations by IsoF

Based on the pro-angiogenic and cellular protective potentials of the IsoF, we investigated some corresponding factor gene expression induced by the IsoF in the RBECs. We found that tumor necrosis factor (TNF)a, angiopoietin-1 receptor (Tie2), and vascular endothelium growth factor (VEGF)-A were significantly up-regulated from 6 to 12 hours stimulation with 1μM IsoF. However, caspase (Casp) 2 and VEGF-C were not altered by the IsoF (Fig. 5).

Discussion

Lipid peroxidative products impact a list of human disorders, such as diabetes, cancer, cardiovascular disease, neurological disorders, and some inflammatory diseases. Lipid peroxidative products, such as aldehydes, act as biomarkers to indicate some disorder processes such as Parkinson’s and Alzheimer’s. Deeper study on lipid peroxidative products would be more important to identify novel signaling

pathways in various human disorders, possible abnormal biomarkers, and to find novel therapeutic approaches[20]. IsoF and IsoP are synthesized under comparable conditions. Higher oxygen intensity favors IsoF production, however, it counts against the IsoP formation[1]. Thus, oxygen tension decides the ratio of IsoP/IsoF [14], which could be applied as an indicator of brain peroxidation [21] and cellular oxidative stress status [1]. However, other pathological properties of the IsoF are still uncovered. Human disorders with abnormal angiogenesis, including atherosclerosis [6], cancer [7, 8], diabetes [9], retinopathy [5] is closely associated with oxidative stress and lipid peroxidation. In those conditions IsoF is advantageous to be produced. To investigate the pathological property of the novel found lipid peroxidative product, IsoF, we performed this project. In this study, the pro-angiogenic activity of the IsoF was verified by endothelial cellular proliferation and migration assays in the RBECs. Experiments to rescue the staurosporine-induced cellular apoptosis revealed the endothelial cellular protective potential of the IsoF. Induction of calcium release and ERK1/2 activation revealed the signaling manner of IsoF. Finally, IsoF clearly induced TNFα, Tie2, and VEGF-A gene up-regulation. Those gene alterations may be involved in the pathological process of IsoF-induced abnormal angiogenesis. These data firstly reveal the pro-angiogenic potential of IsoF. The findings of this study may open a door to investigate the pathogenic activity of the IsoF in other abnormal angiogenic disorders.

IsoP, as another kind of lipid product which was formed through a very similar pathway to IsoF, reveal an activity on vascular regulation. For example, PGE2 and PGI2 act as arterial vasodilation factors [22, 23]. Different PGs isoforms present different regulatory effects on angiogenesis as well. High expression of aldo-keto reductase 1C3 promotes the growth of skin squamous cell carcinoma via inhibition of PGD2, indicating that PGD2 has an anti-angiogenic potential [24]. However,PGF2a is increased and acts as a pro-angiogenic factor in sarcoma [22]. Decrease of PGE2 is associated with angiogenesis and tumor spread in fibroblast tumor [22]. Here, we showed that IsoF present a pro-angiogenic potential. Other activities concerning on vascular regulation by IsoF need to be addressed further.

In this study, we found that 1μM IsoF created maximal biological response to induce RBEC's proliferation. Fessel JP *et al* (1) have measured the concentration of IsoF in rat plasma and indicated that the IsoF concentration of rat plasma is 334 ng/ml, that is equal to 0.571μM (based on the calculation with 585 as the molecular weight of IsoF). Thus, it is reasonable that doubling of this level (1μM) of the IsoF could be enough to induce a pathological response. However, this data only shows the response dose for RBEC (rat brain vascular endothelium). This dose may not be optimal for other endothelium because endothelium has a well-known district specificity[25] as well as different tissues having different physiological levels of IsoF. For example, the IsoF's level in human plasma is 71ng/ml (0.121μM)(1). We recommend doing a dose response assay of the IsoF for different endothelium when performing experiments in different endothelium.

Although the sequence homology is not the same between classes, all GPCRs obtain a universal structure and similar signal transduction mechanism. Particularly, the C-tail of GPCR usually contains either serine or threonine residues. Once they are phosphorylated, the intracellular structure will bind scaffolding proteins called β-arrestins [26] to interrupt G-protein coupling and enlist other proteins, resulting in

signaling complexes activation, including ERK pathway activation, or receptor endocytosis. Thus, IsoF-induced cellular ERK1/2 phosphorylation (Fig. 5) hints that the receptor of the IsoF might be a GPCR.

In phosphatidylinositol signal pathway of GPCR, ligand binds with Gq to activate phospholipase Cs located on the plasma membrane. The lipase hydrolyzes phosphatidylinositol 4,5-bisphosphate into either diacylglycerol or inositol 1,4,5-trisphosphate (IP3). The IP3 associates with the IP3 receptor in the membrane of the smooth endoplasmic reticulum and mitochondria to open Ca^{2+} channels. On the other hand, ligand-binding with Gβγ trigger various ion channels, including N-type voltage-gated Ca^{2+} channels to induce calcium release. [27]. In this study, the Ca^{2+} release by the IsoF (Fig. 4) suggests that the receptor of IsoF could be either a Gq or a Gβγ type GPCR. However, whether a GPCR is the receptor of IsoF need further detailed investigation.

In our previous study, TNFα and Tie2 play a crucial role in the protease-activated receptor 2-induced endothelial angiogenesis via ERK1/2 signaling[28]. Interestingly, here we showed that IsoF not only up-regulates both Tie2 and TNFα, but also activates ERK1/2 signaling, suggesting that the ERK1/2-TNFα-Tie2 axis participates in the pro-angiogenic mechanism of the IsoF. Further, VEGF-A up-regulation by IsoF may be one of the pro-angiogenic mechanism of the IsoF as well.

In this study, we performed Coverslip-removing migration assay(15) to address the pro-angiogenic property of the IsoF. This method is created based on the Wound and Healing migration assay, but it overcomes a weak point of Wound and Healing migration assay where the migration borders are manually defined. This novel method applies a 10mm diameter coverslip that has a clear, constant, and standard borders. In addition, it employs MTT stain method to figure out the migrated cells. This way, the assay is getting easy and mechanical operation without any infection from subjective awareness of operators. In the contrary, manually drawing the migrated area is performed in the Wound and Healing migration assay, which could be impacted by subjective awareness of operators. The operator can easily get bored and fatigued because of the repeated manual works. Due to the 48–72 hours assay processes in Coverslip-removing migration assay, to overcome the impact of results caused by cellular proliferation, mitomicine D is employed to inhibit the cellular proliferation. Indeed, Wound and Healing migration assay has this problem as well.

The limitation of this study is that it did not define whether interfering ERK1/2 affects Tie2, TNFα, or VEGF-A gene/protein expression, to get the direct evidence that the ERK1/2-TNFα-Tie2-VEGF-A axis participate in the IsoF-induced angiogenesis. In addition, lipid peroxidation is closely associated with inflammation. Whether the pro-angiogenic activity of the IsoF is caused due to the promotion of inflammatory reaction needs to be further addressed, because inflammation response is always companied with angiogenic process. Indeed, addressing those questions is already scheduled in our next research plan.

Conclusion

This study first reveals that IsoF have pro-angiogenic potential through induction of endothelial cell proliferation, migration, cellular protection as well as regulation of some pro-angiogenic factors in RBECs. These findings provided preliminary evidence of pro-angiogenic activity of IsoF and some possible mechanisms, which will help scientists recognize the biomedical function of the novel lipid peroxidative product. In addition, it affords a novel mechanism and research direction for biomedical researche concerning peroxidative damaging disorders.

Materials And Methods

Cell culture

Primary RBECs were purchased from Cell Application (San Diego, CA, USA). The cells below p15 were cultured in RBEC Growth Medium (Cell Application) with 10% Growth Supplement (Cell Application) and 1% penicillin and streptomycin (Sigma-Aldrich, Oakville, Ontario, Canada) in a 5% CO_2 humidified atmosphere and 37^{o}C incubator. For function analysis, all the RBECs were pre-starved in RBEC Growth Medium with 2% Growth Supplement for six hours to 24 hours.

Isofuran (IsoF)

IsoF was synthesized by Dr. L. Jackson Roberts' laboratory (Vanderbilt University, Nashville, TN, USA) and provided by Dr. Sylvian Chemtob (Université de Montréal, Montreal, QC, Canada).

Cellular proliferation and viability assay

Cellular proliferation and viability were measured based on the reduction of MTT (Sigma-Aldrich) by mitochondria as previously described [28].

Cellular migration assay

Cellular migration was estimated with Coverslip-removing migration assay method as previously described [29]. In brief, 10mm diameter circle microscope glass coverslip (Thermo Fisher Scientific; Waltham, MA, USA) was sterilized by flame and put onto the bottom of a well in a 24-well dish (Falcon; Corning, New York, USA). RBECs were seeded at the density of 5 x 10^5 cells per well and incubated in 0.5mL of complete RBEC Growth Medium (with 10% Growth Supplement) overnight. To eliminate the impact of cellular proliferation, the cells were treated with 10 mg/ml mitomycin C (Cell Signaling Technology, Danvers, MA, USA) for 30 min at 37°C. The cell-seeded coverslip (face up) was moved onto a well of 12-well dish (Falcon) and incubated in 2ml indicated stimulator containing medium (2% Growth Supplement and 1% penicillin and streptomycin) for 72 hours. 2% Growth Supplement medium alone was used as negative control and 10% Growth Supplement medium was used as positive control. Next, the

cover glass containing the original cells was removed and the remaining cells on the bottom were the migrated cells. The quantity of the migrated cells was estimated by MTT stain (see Cellular proliferation and viability assay).

TUNEL apoptosis assay

Cellular apoptosis was assayed by In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland). In brief, 1 x 10^5 RBECs were seeded on a 12-mm coverslip-containing 24-well culture dish with 500μl per well RBEC complete medium. To induce apoptosis, the cells were treated with 0.6125 μM staurosporine (Sigma) overnight at 37^{o}C incubator, fixed with 200μl l4% formaldehyde (Sigma) for 60 min at room temperature, and washed with 200μl/well PBS buffer. The coverslip was picked up face-up, placed on a support, and the cells on the coverslip were permeabilized with 200μl/well 0.1% Triton X-100 in 0.1% sodium citrate (Permeabilization Solution) for 2 min on ice, washed twice with 200μl/well PBS, TUNEL stained with 50μl/coverslip TUNEL Reaction Mixture (provided from the kit) for 60 min at 37^{o}C while avoiding light. After washing with PBS twice, the cells were stained with 10μg/mL DAPI (Sigma), washed with PBS again, and the coverslip was then embedded on a glass-slide and observed on a 20x magnification lens of CKX53 Fluorescence Microscope (Olympus; Tokyo, Japan). The DAPI (blue)-stained and TUNEL (green)-stained cells were imaged. DNase-treated cells were employed as positive controls. Cells treated with Label Solution instead of TUNEL regent were used as negative controls. The apoptotic rate was calculated by dividing the number of the TUNEL-stained cells to the number of the DAPI-stained cells from at least five random-selected fields.

Western blot

The 1 x 10^5 RBECs in six-well plate were starved with 2% Growth Supplement medium overnight, stimulated with IsoF (final to 1μM), terminated by 300μl pre-cooled lysis buffer at 0, 2, 5, 10, 20, 30min, and harvested in pre-cooled 1.5mL-eppendorf tubes. The cellular sample (supernatant lysate) was obtained by centrifugation at 5000x g for 10min at 4^{o}C. The processes of the Western blot were followed as described previously [28]. The rat antibodies of ERK1/2 and phospho-ERK 1/2 were from Abcam (Cambridge, United Kingdom;Cat.: ab17942; 1/500 dilution folds) and ThermoFisher (Waltham, MA, USA; Cat.: MA5-1574; 1/500 dilution folds), respectively.

Calcium mobility assay

Cellular calcium mobility assay were performed with Fluo-4NW Calcium Assay Kits (Molecular Probes/Thermo Fisher Scientific; Waltham, MA) as previously described (16). In brief, 1 x 10^4 RBECs per well were seeded onto 96-well plate. On the next day, the culture medium was exchanged with 100μl of Dye Loading Solution (provided from the kit) and incubated at 37^{o}C for 30 min. The plate was put into an

EnVision Microplate Screening Fluorescence Reader (PerkinElmer EnSight; Waltham, MA) and started to record. Once the IsoF (final at 1μM) was injected at the 5th second by the pump of the reader, the released Fluo-4NW-labled calcium was measured by the reader at 494 nm excitation and 516 nm emission for 80 seconds.

RT-PCR

RT-PCR procedure was described previously [30]. Primers were synthesized from Alpha DNA (Sangon Biotech, Shanghai, China). QuantumRNA™ 18S Internal Standards (Thermo Fisher) was employed as primers for house-keeping gene reference detection. Corresponding primer sequences are listed in Supplemental table 1.

Table 1

Primer sequences for qRT-PCR analysis

TargetRNA/Gene RNA/Gene	Forward primer	Revers primer
rTNFa	CTCAAAACTCGAGTGACAAGC	CCGTGATGTCTAAGTACTTGG
rTie2	TGGAGAAGGACATCCTGGAC	GTTCCCAGGCACTTTGATGT
rCasp2	GAATACCAAACGGGGTTCCT	GGGAACAGGTAGAGCTGCTG
rVEGF-A	GGAGGTGGTGGTACCTCTGA	GATCTGCATTCGGACTTGGT
rVEGF-C	GGGCAGAATCATCACGAAGT	ATCTGCATGGTGATGTTGGA

Abbreviations

2-ALPI: 2-arachidonoyl lysophosphatidylinositol; CB1R: cannabinoids receptor 1; CCl4: carbon tetrachloride; ERK: extracellular regulated protein kinases; GPCR: G protein-coupled receptor; GPR55: G protein-coupled receptor 55; LPI: Lysophosphatidylinositol; RBMVEC: rat brain microvascular endothelial cells; IsoF: Isofuran; IsoP: isoprostaine.

Declarations

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Authors'contributions

Tang Zhu designed research and edited the manuscript. Jingxia Zhang performed most of the experiments, analysed the data, and drafted the manuscript. Xiangjiang Wang participated in the design of experiments and contributed to the Calcium mobility assays. Zhiping Lin analyzed data analysis and edited the manuscript. All authors have approved the final article.

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Availability of data and materials

All data generated and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable since the study did not involve in any animals or human samples.

Consent for publication

Not applicable.

Competing interests

No conflict of interest exists between the authors.

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Figures

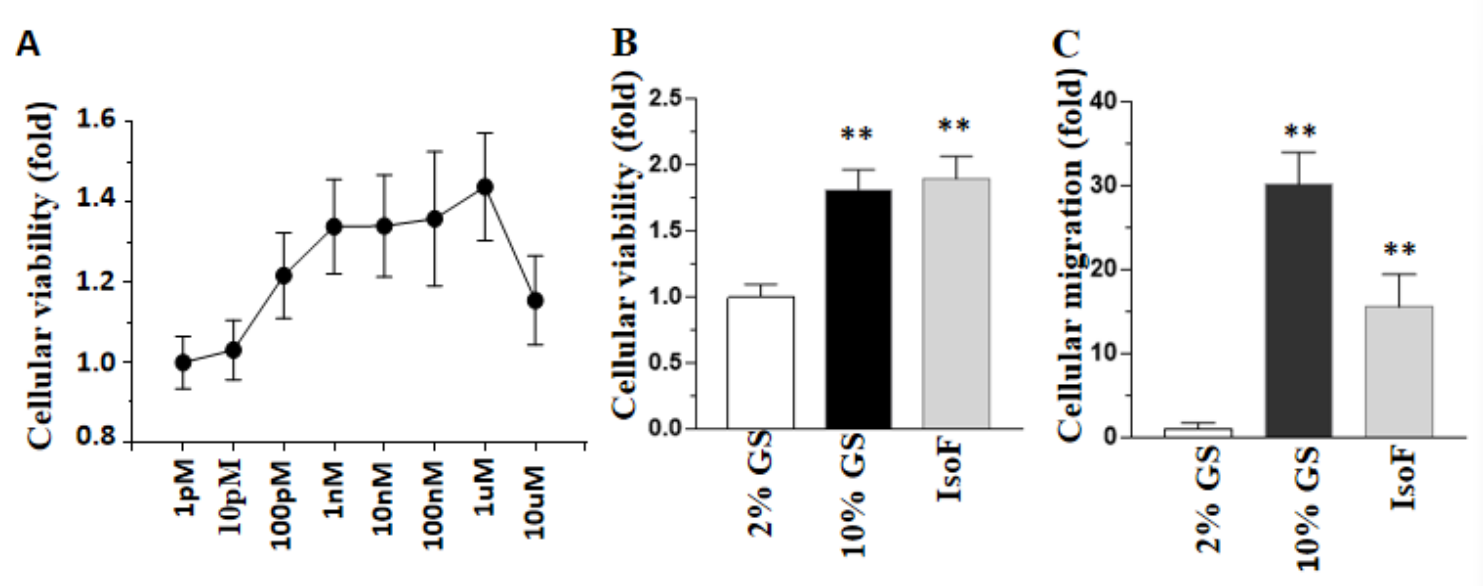

Figure 1

Pro-angiogenic activity of IsoF. A. The optimal dose of the IsoF was detected in a dose-response assay (from 1pM to 10μM) of cellular viability by MTT stain in the RBECs. Cellular proliferation (**B**) and migration (**C**) were examined by MTT assay. (**B**) and Coverslip-removing migration assay (**C**), respectively, in the RBECs stimulated with 2% Growth Supplement medium (negative controls), 10% Growth Supplement medium (positive controls), and 1μM IsoF. The cellular viability rate at minimal dose (1pM) and the negative controls (2% Growth Supplement medium) of cellular proliferation and migration assay were normalized as one. Each data point was obtained from five independent assays. ** indicates that the $p<0.01$ compared to 2% Growth Supplement medium-treated cells.

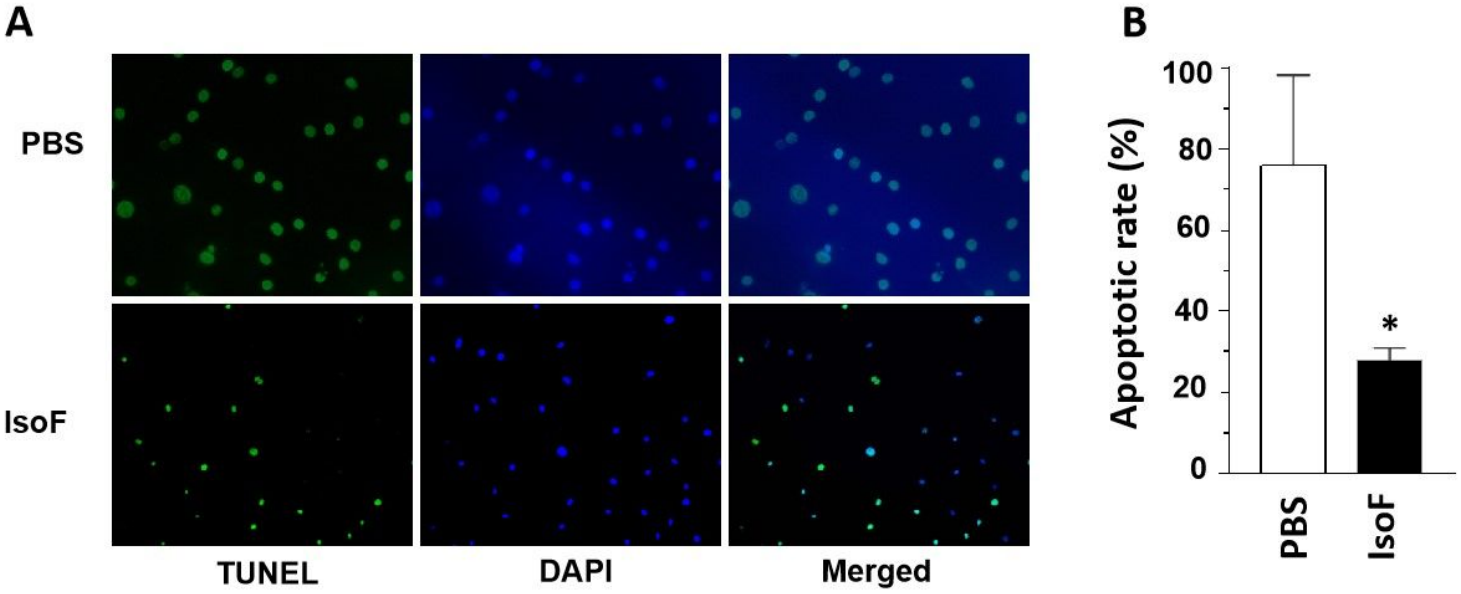

Figure 2

Anti-apoptotic activity of the IsoF. A. Cellular apoptosis is induced by 0.6125 μM staurosporine in the RBECs overnight. The cellular protective potential of the IsoF is detected in the cells pre-treated with 1μM IsoF and then induced by the staurosporine. PBS pre-treated cells (replacing the IsoF) are used as positive controls. Apoptotic cells are detected by TUNEL (green) stain. The survival cells are stained by DAPI (blue). The apoptotic cells are defined as the cells either stained by TUNEL or DAPI (merged). **B.** The apoptosis rate of the cells treated with PBS or 1μM IsoF are calculated by the TUNEL-stained cells divided by the total DAPI-stained cells. Each data point is obtained from five independent assay. * indicates that the $p<0.05$ compared to 2% PBS-treated cells.

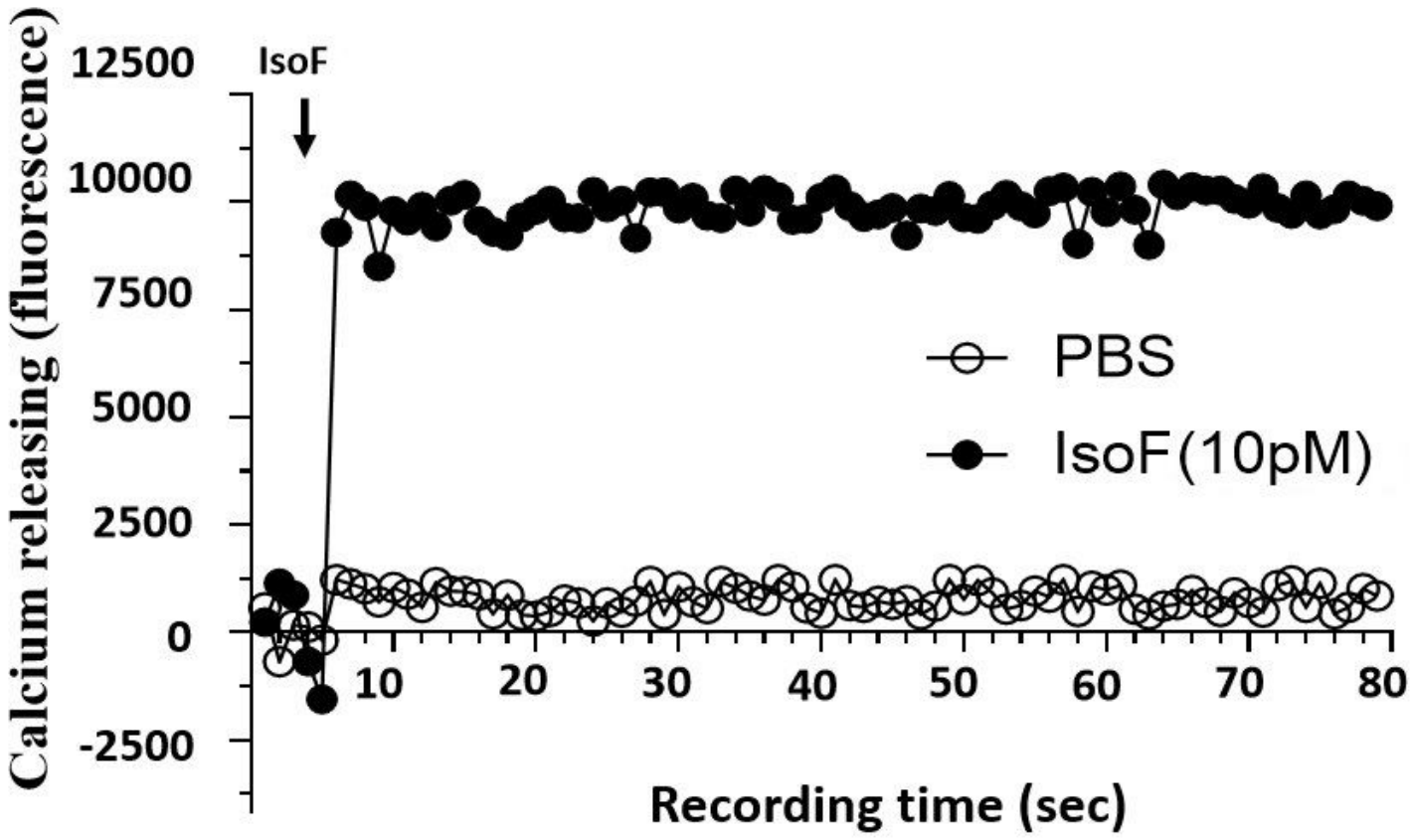

Figure 3

IsoF induced cellular calcium release in RBECs. RBECs are stained with Dye Loading Solution (provided from the Fluo-4NW Calcium Assay Kits) for 30min at 37°C, stimulated by auto-injection with IsoF (black circle; final concentration at 1μM; indicated in the figure), and the fluorescence Fluo-4NW-labled calcium released from cells is immediately detected by EnVision microplate Screening Fluorescence Reader with 494 nm excitation and 516 nm emission for 80 seconds. Same value of PBS (open circle) is added to replace the IsoF as negative controls. Each data point was obtained from five independent assays.

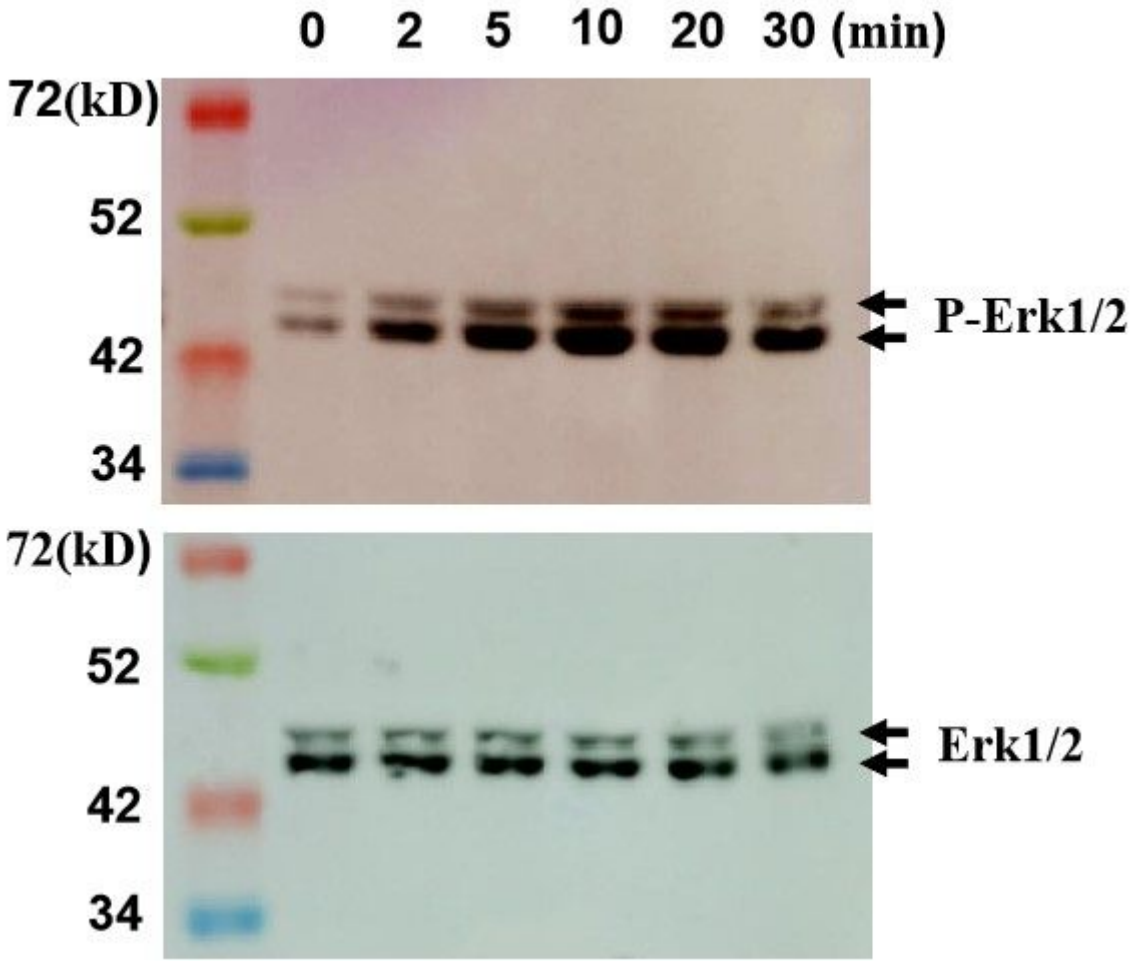

Figure 4

IsoF activates ERK1/2 phosphorylation in RBECs. ERK1/2 activation (phosphorylation, P-ERK1/2; up panel) is detected by Western blot in a time-course (0-30min) after stimulation with 1μM IsoF in the RBECs. The loading amount of the samples is probed with general ERK1/2 antibody (ERK1/2; lower panel) in the same membrane after tripping. Protein markers are indicated on the left. The sample harvesting times (min) are shown at the top. Each data point was obtained from five independent assays.

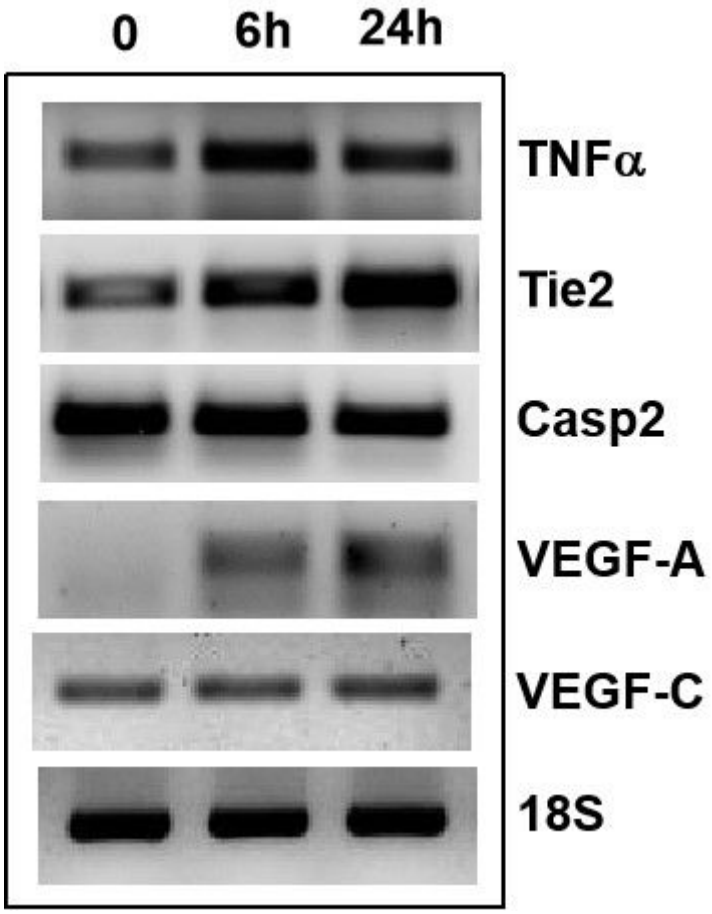

Figure 5

IsoF induced gene expression in RBECs. The images show that *TNFa, Ties, Casp2, VEGF-A,* and *VEGF-C* gene expression are analyzed by RT-PCR in the RBECs before (0) and after stimulation with 1μM IsoF for 6h or 24h. Each data point was obtained from five independent assays.