Nitric oxide (NO) is an important signaling molecule involved in many physiological processes. Above all, NO is a dominant vasodilator released from endothelial cells and identified as the nature of endothelium-derived relaxant factor so called, which regulates blood pressures. In addition, NO prevents endothelial apoptosis, platelet aggregation and has tissue-protective actions against such proinflammatory and proatherogenic stimulations. NO is produced from arginine and nitrate in normal human serum are around 6.6 and 34 µM, respectively. Nitric oxide (NO) is an important signaling molecule involved in many physiological processes. Above all, NO is a dominant vasodilator released from endothelial cells and identified as the nature of endothelium-derived relaxant factor so called, which regulates blood pressures. In addition, NO prevents endothelial apoptosis, platelet aggregation and has tissue-protective actions against such proinflammatory and proatherogenic stimulations. NO is produced from arginine and nitrate in normal human serum are around 6.6 and 34 µM, respectively. Nitric oxide (NO) is an important signaling molecule involved in many physiological processes. Above all, NO is a dominant vasodilator released from endothelial cells and identified as the nature of endothelium-derived relaxant factor so called, which regulates blood pressures. In addition, NO prevents endothelial apoptosis, platelet aggregation and has tissue-protective actions against such proinflammatory and proatherogenic stimulations. NO is produced from arginine and nitrate in normal human serum are around 6.6 and 34 µM, respectively. Nitric oxide (NO) is an important signaling molecule involved in many physiological processes. Above all, NO is a dominant vasodilator released from endothelial cells and identified as the nature of endothelium-derived relaxant factor so called, which regulates blood pressures. In addition, NO prevents endothelial apoptosis, platelet aggregation and has tissue-protective actions against such proinflammatory and proatherogenic stimulations. NO is produced from arginine and nitrate in normal human serum are around 6.6 and 34 µM, respectively.
AMPK activation has been shown to phosphorylate and activate eNOS, which is widely accepted as one of the important regulatory mechanisms of eNOS activity and the following vasodilation.37)

In the present study, we investigated the effects of pharmacological stimulation by nitrite on eNOS and related molecules in human glomerular endothelial cells (HGEC) to unveil possible molecular mechanisms underlying the glomerular protective actions of nitrite, some of which we have previously reported. Here we demonstrate that nitrite activates the AMPK–eNOS pathway, which should be involved in the beneficial effects of nitrites.

METHODS

Materials and Reagents  All reagents were analytic grade and obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan) or Kanto Chemical (Tokyo, Japan) unless otherwise stated. Normal human glomerular endothelial cells (HGEC) at 5 passages were obtained from Cell Systems (Kirkland, WA, U.S.A.).

Cell Culture and Stimulations  HGEC were cultured as previously described elsewhere.38) All the experiments were performed using the cells within 9 passages (3–4 passages after acquisition). In brief, HGEC were cultured in CS-C complete medium containing 10% serum (Cell Systems) in dishes coated with type I collagen (Iwaki Glass, Tokyo, Japan). The cells were serum-starved for four hours in serum-free Dulbecco’s minimum essential medium containing 0.1% bovine serum albumin (BSA), and then stimulated in Krebs–Ringer containing 20mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (Hepes)-Na and 0.1% BSA (pH 7.4).34)

Western Blotting Analysis  The stimulated cells were lysed in the buffer containing Triton X-100.33,36,39,40) Twenty or thirty microgram of protein per each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gel (Bio-Rad). Antibodies for eNOS (#9572), pSrc1177-eNOS (#9571), acetyl-CoA carboxylase (ACC) (#3662), pSer79-ACC (#3661), Akt and pThr308-Akt (#8205), AMPK alpha (#2532) or pThr172-AMPK alpha (#8308) were from Cell Signaling Technology (Danvers, MA, U.S.A.) and used in 1 : 500 or 1 : 1000 dilution. Anti-Fc fragment of rabbit immunoglobulin G (IgG) conjugated with peroxidase was from Wako and used in 1 : 5000 dilution. ECL reagents (GE Healthcare, Little Chalfont, U.K.) and a LAS-4000 image analyzer (FUJIFILM, Tokyo, Japan) were used for detection and quantification as described previously.33,36,39,40)

Measurement of ATP in HGEC  HGEC were seeded and grown to confluent in a collagen-coated 96-well tissue culture plate. Following the stimulation, ATP levels in the cells were determined by D-luciferin-luciferase luminescence method using 1-step ATPlite reagent (PerkinElmer, Inc., Waltham, MA, U.S.A.).

RESULTS

Nitrite Stimulation Leads to eNOS Phosphorylation without Akt Activation  To clarify whether eNOS is involved in the pharmacological effects of nitrite, we determined phosphorylation levels of serine 1177 residue of eNOS, the most typical index of its activity. The eNOS phosphorylation was increased by nitrite stimulation in a dose-responsive manner in HGEC (Fig. 1A). Akt is a typical upstream kinase of eNOS and regulates activity of eNOS in endothelial cells. We investigated Akt phosphorylation to identify the kinase which phosphorylates eNOS in response to nitrite stimulation. The phosphorylation level of threonine 308 residue of Akt is well known to correlate with its activity. In contrast to facilitation of its phosphorylation by vascular endothelial growth factor (VEGF) stimulation, neither phosphorylation nor expression levels of Akt were changed by nitrite stimulation within the range of the concentration between 1–30μM, which are similar conditions where eNOS phosphorylation was observed (Fig. 1B). An activator of AMPK, 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR) did not increase the Akt phosphorylation, either.

Nitrite Stimulation Evokes AMPK Activation  AMPK is also known to phosphorylate and activate eNOS in endothelial cells. In contrast to Akt, nitrite stimulation increased phosphorylation level of threonine 172 residue of AMPK α, which is a reliable indication of the activity, at the concentration which could induce eNOS phosphorylation (Fig. 2A). The phosphorylation level of ACC serine 89 residue, which is a direct substrate of AMPK, was also increased by nitrite stimulation in accordance with the AMPK phosphorylation (Fig. 2B). Nitrite is subject to change into nitrate under some conditions like acid. However, stimulation by nitrate did not alter phosphorylation levels of AMPK nor eNOS in contrast to nitrite (Fig. 2C).
AMPK Lies Upstream to the eNOS Activation

AMPK and NO production are known to potentially cause mutual stimulation. AMPK is an upstream kinase of eNOS, while NO is a potent activator of AMPK. Therefore we investigated the effect of a typical cell permeable NO quencher, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), on AMPK phosphorylation by nitrite stimulation to clarify which molecule the genuine upstream regulator is. Pretreatment of carboxy-PTIO did not affect the increase in AMPK phosphorylation by nitrite stimulation (Fig. 3A).

We then determined the effect of an AMPK-specific inhibitor, BML275, on eNOS phosphorylation to clarify whether AMPK lies upstream of eNOS in the nitrite action. Pretreatment of BML275 almost completely inhibited the increase in AMPK phosphorylation evoked by nitrite stimulation (Figs. 3B, C).

Nitrite Stimulation Activates AMPK in an Intracellular Energy-Dependent Manner

Then we measured ATP concentration in order to determine whether the AMPK activa-
tion by nitrite stimulation is caused by energy depletion or its direct action on AMPK. The intracellular ATP levels were decreased by nitrite stimulation as time went on (Fig. 4A). Pretreatment of BML275 did not affect the ATP reduction by nitrite, though ATP level was decreased solely by BML275, demonstrating that the decrease in ATP level was not caused by AMPK activation (Fig. 4B).

DISCUSSION

This is the first report demonstrating that stimulation by nitrite leads to eNOS activation in endothelial cells. We and others have reported that orally administered nitrite or nitrate, which should be converted to nitrite, is nonenzymatically reduced into NO independently of NOS in vivo.10–19) NOS requires oxygen to generate NO from arginine, and NO production by NOS should be diminished in ischemic conditions. Therefore, the nonenzymatic NO production from nitrite is recognized as a significant vasoprotective mechanism especially in ischemic tissues. However, physiological relevance of nitrite in uninjured tissues has been obscure even though there are substantial amounts of nitrite throughout our bodies. Our result that nitrite is a potent activator of eNOS demonstrates that nitrite should intensify NO production where oxygen is available and eNOS can generate NO. In normoxic normal tissues, nitrite is supposed to enhance vasodilation using eNOS (Fig. 1A).

Importantly, we found that nitrite activates AMPK in endothelial cells (Fig. 2A). A report based on the similar concept was released recently. Mo et al. demonstrated that stimulation by nitrite for three hours promotes phosphorylation of AMPK using primary rat smooth muscle cells cultured under severe hypoxia at 1% O2.41) They also showed increase in the number of mitochondria and suggested the relevance of Sirt1-peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) pathway lying downstream of AMPK. This report strongly supports our notion that nitrite is a potent activator of AMPK, although they observed a series of these phenomena only in the severe hypoxic conditions. Actually, they did not find a change in mitochondrial number or AMPK phosphorylation under normoxia in contrast to our observations. Even though the reason of the difference is uncertain at the moment, we speculate that the primary rat smooth muscle cells may be more resistant to AMPK-stimulating stresses as even the severe hypoxic stimulation for three hours did not cause AMPK activation in the cells.50) Sensitivity to nitrite may also vary among the types of the cells. In addition, the AMPK-activating effect of nitrite appears to be most potent at the concentration around 10 µM in HGEC, which suggests possible existence of non-specific counteractive effects.

The serine 1177 residue of eNOS has been shown to be phosphorylated by Akt as well as the aforementioned AMPK.37–40) Phosphoinositide 3-kinase (PI3K)–Akt pathway is widely accepted as a central player in growth factor signal transductions including insulin signals, regulating eNOS in endothelial cells. However, Akt is not supposed to be involved in the eNOS activation by nitrite as we did not find any change in the phosphorylation levels of Akt under the nitrite stimulation in comparable conditions to that could promote eNOS phosphorylation (Fig. 1B). Therefore, nitrite is suggested to activate eNOS in not an Akt but an AMPK-dependent manner.

A close relationship has been suggested between AMPK and eNOS. As mentioned above, AMPK is known to phosphorylate and activate eNOS directly. However, NO was also reported to lie conversely upstream of AMPK, and NO donor induces AMPK activation.45) Thus we determined the effects of NOS and AMPK inhibition to identify the molecule which is an upstream regulator since nitrite is a potent donor of NO. We further studied the effect of carboxy-PTIO as we and others have demonstrated that NO can be formed from nitrite without NOS, which did not affect the AMPK phosphorylation (Fig. 3A). NO did not seem to mediate AMPK activation by nitrite stimulation. In contrast, an inhibitor of AMPK, BML275 completely blocked eNOS phosphorylation by nitrite (Figs. 3B, C). Therefore, AMPK should be an upstream enzyme regulating eNOS.

Then what kind of mechanism is involved in the AMPK activation by nitrite? Nitrite and its derivatives have been shown to inhibit cellular respiration by chemical modulation of molecules in respiratory chain, which could result in suppression of ATP production.46) We confirmed acute decrease in ATP levels under nitrite stimulation, suggesting that AMPK activation by nitrite should be energy level-dependent even though the exact target molecule of nitrosylation responsible for the AMPK activation is to be determined at the moment (Fig. 4A). Though the nitrite-stimulated ATP reduction may not appear strong, intracellular ATP level is usually strictly maintained homeostatically, and nitrite is supposed to change the energy status strongly enough to cause AMPK activation.

Several recent reports showed that dietary nitrate supplementation increases exercise performance in human studies.47–51) AMPK plays important roles in improving exercise tolerance as well as acute metabolic responses in terms of skeletal muscle contraction.28,52) They all suggest the importance of nitrate–nitrite-derived NO production in the amelioration of muscle energetics. Our observations suggest the significance of nitrite-stimulated AMPK in the nitrate-evoked improvement of exercise performances. In addition, dietary supplementation of nitrate has been shown to prevent metabolic disorders including glucose intolerance and hyperlipidemia in eNOS-deficient mice without demonstrating the relevant molecular mechanisms.53) Nitrate did not change phosphorylation levels of eNOS, AMPK or ACC in contrast to nitrite, which indicates that nitrate itself is inactive (Fig. 2C). AMPK activation by nitrite we demonstrated here may be also involved in the metabolic effects of dietary nitrate (Fig. 4C). In fact, we found nitrite-evoked increase in ACC phosphorylation, which should in turn increase lipid oxidation and decrease lipogenesis (Fig. 2B). Furthermore, nitrite may be effective on other diseases as AMPK has pleiotropic beneficial effects on metabolic, cardiovascular, renal and neural disorders, although it remains to be determined whether AMPK is activated by nitrite comparably in other tissues such as skeletal muscles.

It is difficult to evaluate eNOS-dependent and/or -independent NO production in the presence of nitrite or nitrate because NO is usually quantified as a form of nitrite with Griess reagents. Thus we could not confirm that the carboxy-PTIO completely diminished NO in our experimental conditions. However, the eNOS and AMPK phosphorylation by nitrite stimulation did not occur in acidic milieu around pH 7.0–6.6,
where nitrite is supposed to be decomposed into NO and nitrate by disproportionation (data not shown), supporting our idea that nitrite activates AMPK–eNOS pathways independently of nitrate or NO. Further study using electron paramagnetic resonance apparatus with isotopic labels will be required to directly demonstrate the enhancement of eNOS-dependent NO production by nitrite.

A series of derivatives of nitrite or nitrate have been traditionally used clinically to treat such coronary artery diseases as angina. AMPK activation by nitrite which we found here, in addition to the NO production, may be involved in the cardioprotective actions of the alkyl nitrates such as amyl nitrate, which are currently in use clinically.

Plants utilize nitrates and nitrites as essential nutrients in nature. Thus nitrates and ammonium salts are used as inorganic fertilizers. Abundant nitrites and nitrates in vegetables are produced naturally. Thus nitrates and ammonium salts are used as inorganic fertilizers. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the coronary arteries.

We adopted HGEC in the current study since we have reported several studies using this cultured cells in addition to the HGEC as a normal endothelial cells from human glomeruli. However, nitrite is supposed to facilitate eNOS-mediated NO production also in other endothelial cells, which is to be confirmed in the future studies.

In conclusion, our results suggested that nitrite should be an activator of AMPK–eNOS pathway in endothelial cells. Nitrite activates AMPK principally in a NO independent but energy status dependent manner. Nitrite is hypothesized to increase NO production by activating AMPK followed by eNOS activation in addition to non-enzymatic reduction previously reported. The AMPK–eNOS activation by nitrite should be a possible molecular mechanism underlying vascular and renal protective effects of nitrite and nitrate. Nitrite may harbor more multipotent beneficial effects as an AMPK activator.

Acknowledgment We thank Ms. Kazuyo Fukumoto and Ms. Kayoko Miyoshi for secretarial assistance, Ms. Mayumi Ozawa for checking the manuscript and Drs. Kazuhiro Tsutsumi, Shinji Abe, Satoko Nakanishi, Taro Toyota and Tatsuya Hayashi for helpful advice. This work was supported by scientific research fund of the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant from the Ito Foundation and the Regional Innovation Cluster Program of Japan.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


