The expression of soluble guanylate cyclase in the vasculature of rat skeletal muscle

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Introduction

Nitric oxide (NO), an unstable free radical, freely diffuses across biological membranes and interacts with its targets to mediate downstream effects. NO is generated from L-arginine by one of the three nitric oxide synthase (NOS) enzymes that are found in various tissues: neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), and inducible NOS (iNOS, NOS2) (Krumenacker et al., 2004). NO performs various roles in a wide range of physiological processes such as neurotransmission, signal transduction, and the regulation of muscle contraction.

The roles of NO in the vertebrate skeletal musculature have been studied not only in normal healthy conditions but also in pathological conditions such as muscular dystrophy and crush injury. NO contributes to the contractile function of muscle fibers, resting potentials of cell membranes, exercise, glucose uptake, blood flow in skeletal muscle, tissue respiration, injury, myoblast differentiation and muscular dystrophy (Stamler and Meissner, 2001). Many studies on NO in the skeletal musculature have demonstrated the distributions of the NOS enzymes. Under normal conditions, nNOS is expressed at the muscle membrane of type II (fast twitch) fibers (Kobzik et al., 1994) and the neuromuscular junction (Kusner and Kaminski, 1996). eNOS expression correlates with mitochondrial contents shown by histochemical stains for succinate dehydrogenase (Kobzik et al., 1995), and is present within mitochondrial preparations of the skeletal musculature (Bates et al., 1996). Although iNOS is absent or expressed at a low level in the normal skeletal musculature (Stamler and Meissner, 2001), its level increases in patients with chronic heart failure (Riede UN et al., 1998) and myopathy (Tews and Goebel, 1998).
NO primarily activates soluble guanylate cyclase (sGC) in target cells (Hofmann, 2000; Krumenacker et al., 2004; Koesling et al., 2004; Pyriouch and Papapetropoulos, 2005). sGC comprises α and β subunits in vivo, and produces cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in this signal transduction system. cGMP acts as a second messenger in cells by regulating cGMP-dependent protein kinase or driving cyclic nucleotide-dependent ion channels (Hofmann, 2000; Pyriouch and Papapetropoulos, 2005). There have been several reports regarding the sGC distributions in the skeletal musculature. Schoser and Behrends (2001) found that sGCα2 and the α1/β1 subunits exist in the sarcolemma and neuromuscular junction. Arterioles in the musculature contain sGCα2 and α1/β1 subunits, while venules contain only the sGCα2 subunit. Buchwalow et al. (2005) reported that sGC coexists with the three NOSs in the sarcolemma and subsarcolemma, although the subtypes were not described. Feussner et al. (2001) studied sGC distributions in most of the mammalian skeletal musculature and reported that positive immunoreactions for sGCβ1 and β2 subunits are located in the myotendinous junctions and non-junctional sarcolemma, and that sGCα1 immunoreactivity can be observed in endplates and myotendinous junctions.

sGC subtypes exist in the smooth musculature of arteries and venules of the skeletal musculature (Schoser and Behrends, 2001) and are responsive to NO derived from eNOS, as is known for various vessel types (Krumenacker et al., 2004). NO derived from nNOS expressed in skeletal muscle cells has also been shown to play an important role in the regulation of the blood flow during exercise by modulating adrenergic vasoconstriction (Thomas et al., 1998). Kobayashi et al. (2008) studied muscle fatigue after mild exercise using a pathological mouse model and found that sarcolemmal-localized nNOS deficiency generated exercise-induced narrowing of the vasculature.

To understand the mechanisms of NO function in blood flow regulation of the skeletal musculature, we sought to identify cells that express sGCα1 and β1 because α1 and β1 dimers are abundant in vivo and can act as an active enzyme (Koesling et al., 2004). We found specific distributions of sGC isoforms in the skeletal muscle vascular system of rats using confocal microscopy and electron microscopy; here, we describe the sGC-immunopositive cells.

Materials and Methods

Animals

Adult Wistar rats aged 5–6 weeks, weighing about 300 g (Japan SLC, Shizuoka), were used in this study. The use and treatment of animals followed the Guidelines for Animal Experiments, University of Fukui Faculty of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering. All rats were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and then tissue blocks of the thigh muscles (M. quadriceps femoris, M. hamstrings, M. adductors) were dissected.

Antibodies

For immunochemical analysis, we used the following antibodies: rabbit anti-soluble guanylate cyclase α1 (sGCα1, G4280; Sigma, MO, USA), rabbit anti-soluble guanylate cyclase β1 (sGCβ1, G4405; Sigma), guinea pig anti-soluble guanylate cyclase β1 (SG6), mouse anti-dystrophin (NCL-DYS2; Novocastra Lab. Ltd. UK), rabbit anti-Ng chondroitin sulfate proteoglycan (AB5320; Chemicon International Inc., CA, USA), monoclonal mouse anti-synaptophysin (M0776; DAKO, Denmark), and monoclonal anti-a-smooth muscle actin (A5228; Sigma). To generate a polyclonal antibody (Iino et al., 2007) specific for sGCβ1 (SG6), a corresponding peptide to residues 606-619 of rat, RKNTGTEETNQDEN, was synthesized (In vitro Japan, Tokyo) and coupled to keyhole limpet hemocyanin. Polyclonal antibodies were prepared by immunizing a female guinea pig (Japan SLC) with the peptide in complete Freund’s adjuvant (DIFCO, MI, USA) and subsequently boosted several times with the same peptide in incomplete Freund’s adjuvant (DIFCO). The specific peptide antibody (SG6) was obtained by affinity chromatography using the appropriate peptide immobilized on formil-cellulofine (Seikagaku Corporation, Tokyo). To specify the immunoreactivity of SG6, the antibody was preabsorbed with the synthesized peptide.

Immunoblot analysis

Muscular tissues were homogenized in 20 mM Tris HCl (pH 7.5), 1 mM ethylene diamine tetra-acetic acid, and 1 mM phenyl methyl sulfonyl fluoride. The homogenates were centrifuged at 1000 × g at 4°C for 30 min, and the supernatants were collected. Proteins were separated by sodium dodecyl sulphate-polyacrylamide
gel electrophoresis using a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA, USA). Membranes were blocked with 5% fat-free dry milk in PBS containing 0.05% Tween-20 and then incubated with rabbit anti-sGCα1 (1:5000), rabbit anti-sGCβ1 (1:5000), or guinea pig anti-sGCβ1 (SG6, 1:1000) antibody solutions. Immune complexes were visualized using a chemiluminescence system (ECL Plus, GE Healthcare UK Ltd., England) with HRP-conjugated anti-IgG.

**Immunofluorescence microscopy**

We used seven rats for cryostat studies. Five rats were perfused via the left ventricle with saline followed by Zamboni’s fixative (2% paraformaldehyde prepared in a 1.5% saturated picric acid solution, 0.1 M phosphate buffer, pH 7.3). Two rats were injected via the right carotid vein, 10 min before perfusion, with FITC conjugated *Lycopersicon esculentum* Lectin (tomato lectin) (FL-1171, Vector, CA, USA). The dissected thigh musculature was fixed with Zamboni’s fixative for 4 h at room temperature. Following fixation, tissues were washed with PBS, immersed in 30% sucrose containing PBS, and embedded in O.C.T. Compound (Sakura Finetechical Co., Tokyo) before being quickly frozen. For confocal laser scanning microscopy, cryostat sections were cut at a 10–20 μm thickness using a Leica CM3050 cryostat (Leica Microsystems, Germany) and collected on poly-L-lysine-coated glass slides. Sections were preincubated with normal donkey serum (5% in PBS) for 1 h before incubation with anti-sGCα1 (1:1000), anti-sGCβ1 (1:1000), SG6 (1:1000), anti-dystrophin (1:1000), anti-NG2 (1:3000), anti-synaptophysin (1:1000), or anti-α-smooth muscle actin (1:1000) antibodies at room temperature overnight. After incubation with the primary antibodies, sections were washed with PBS for at least 1 h before incubation with the secondary antibodies (Alexa Fluor 488 or 555 -coupled donkey anti-IgG, Molecular Probes, OR, USA, 1:500 in PBS) for 1 h at room temperature. After washing with PBS, the specimens were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Molecular Probes) and mounted with PermaFluor Aqueous Mounting Medium (Thermo Electron Corporation, MA, USA). Double-labeled immunofluorescence images were examined using a Leica TCS-SP2 confocal microscope (Leica Microsystems) with excitation wavelengths of 405 nm, 488 nm, and 543 nm. Images were collected and evaluated using Leica Confocal Software (Leica Microsystems). Adobe Photoshop CS2 (Adobe Systems, CA, USA) was used to compose the final plates.

**Immunoelectron microscopy**

The rat thigh musculature was fixed with Zamboni’s fixative plus 0.1% glutaraldehyde for 1 h. The tissues were immersed in Zamboni’s fixative for an additional 3 h at room temperature. Tissues were then cut using a cryostat at 12 μm, and mounted on poly-L-lysine coated slides. After washing with PBS, sections were incubated for 1 h in 10% normal goat serum, and then reacted with rabbit anti-sGCβ1 (1:3000) for 24 h at 4°C. The sections were washed 3 times with PBS, and further reacted with biotinylated goat anti-rabbit IgG (1:200 with PBS, Vector) for 2 h. After washing with PBS, they were reacted with an avidin-biotin-peroxidase complex (ABC Elite kit, Vector) for 2 h. The sections were incubated for several minutes with a solution containing 0.03% dianinobenzidine, 0.005% H2O2 in 0.1 M Tris-HCl, pH 7.6. After colour development in dianinobenzidine solution, the specimens were post-fixed in 1% OsO4 in a 0.1 M phosphate buffer, pH 7.4 for 1 h, block-stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon 812 (Oken, Tokyo). Ultrathin sections were examined using an H-7650 electron microscope (Hitachi, Tokyo).

**Results**

**Immoblot analysis**

Immoblots using rabbit anti-sGCα1 and rabbit anti-sGCβ1 antibodies demonstrated the presence of specific immunoreactive bands in rat muscle tissues at approximately 77 kDa (sGCα1) and 68 kDa (sGCβ1) (Fig. 1). The newly generated guinea pig anti-sGCβ1 (SG6) antibody also showed a single specific band, similar to the rabbit anti-sGCβ1 antibody.

**Immunofluorescence microscopy**

To identify sGCβ1-immunopositive cells, we examined the relationship between sGCβ1-immunopositive cells and sarcolemma stained with the anti-dystrophin antibody. In cross sections of muscle tissues, sGCβ1-immunopositive cells that had a round or an oval cell body (6.5–9.2 μm diameter) with long processes were observed at the outer side of the sarcolemma (Fig. 2A, C). sGCβ1-immunopositive cells were distributed in the space around the skeletal muscle cells. Previous reports indicated that sGC immunoreactivity was distributed in the sarcolemma, especially the neuromuscular junction (Schoser and Behrends, 2001). We used double-staining
immunohistochemistry with sGCβ1 and synaptophysin, a marker of nerve terminals (Torri-Tarelli et al., 1990), to detect the neuromuscular junction. The sarcolemma and neuromuscular junctions did not show sGCβ1 immunoreactivity (Fig. 2C).

We then searched for any relationship between sGCβ1-immunopositive cells and the vasculature using FITC-conjugated tomato lectin, a marker of vascular endothelium (Morikawa et al., 2002). All sGCβ1-immunopositive cells were observed in the vicinity of tomato lectin-labelled blood vessels, especially the capillaries (Fig. 2B). Both the cell bodies and processes of sGCβ1-immunopositive cells were associated with blood vessels. All capillaries that we observed in this study were associated with sGCβ1-immunopositive cells. Although the vascular wall of large diameter vessels showed sGCβ1 immunoreactivity, their morphological characteristics differed from the sGCβ1-immunopositive cells associated with capillaries. sGCβ1-immunopositive cells in the large diameter vessels showed α-smooth muscle actin immunoreactivity (Fig. 2D, D').

We prepared a new sGCβ1 antibody by immunizing a guinea pig and used this to confirm the sGCβ1 immunoreactivity revealed by the rabbit antibody purchased from Sigma. The newly generated sGCβ1 antibody (SG6) detected the same type of cells as the rabbit sGCβ1 antibody in the muscular tissues (Fig. 3A, B). The immunoreactivity revealed by the SG6 antibody was not observed after antibody preincubation with an immunizing peptide (not shown). Using double-staining immunohistochemistry, both sGCβ1 antibodies labeled the same cells in the muscle tissues (Fig. 3A, B).

In the longitudinal sections of muscle fibers, sGCβ1-immunopositive cells displayed long cell bodies and several cytoplasmic processes (Fig. 3B). These processes ran parallel to the muscle fibers (Fig. 3B). The cell processes of sGCβ1-immunopositive cells were 25–50 μm long, the longest process that we could trace being 110 μm in length. sGCβ1-immunopositive cells adjacent to each other formed cellular networks with their processes. Using anti-sGCα1 and anti-sGCβ1 (SG6) antibodies, we observed the two sGC isoforms in the muscle tissues (Fig. 3C, D). In cross sections, anti-sGCα1 and anti-sGCβ1 immunoreactivities were observed in the same cells. Longitudinal sections showed that double-immunopositive cells had elongated cell bodies and cytoplasmic processes extending parallel to muscle fibers.

The morphologies and locations of sGCβ1-immunopositive cells strongly suggested that these cells were capillary pericytes, which are vascular mural cells around the endothelium (Shepro and Morel, 1993; Fujiwara et al., 1999; Higuchi et al., 2000; Armulik et al., 2005). We then used rabbit anti-NG2 chondroitin sulfate proteoglycan as a marker of pericytes (Ozerdem et al., 2001; Armulik et al., 2005) (Fig. 3E, F). Double-staining immunohistochemistry using anti-sGCβ1 and anti-NG2 antibodies revealed that all sGCβ1-immunopositive cells showed NG2 immunoreactivity. NG2 immunoreactivity showed diffuse distributions on the cell surfaces of the cell bodies and processes, as expected for NG2 as a marker of chondroitin sulfate proteoglycan. The sGCβ1 immunoreactivity in the pericytes was distributed in the cytoplasm of cell bodies and processes, surrounded by NG2-immunopositive cell surfaces.

**Immunoelectron microscopy**

Electron microscopic studies were also performed to verify the cellular identities and study the ultrastructural features of sGC-immunopositive cells in the muscle tissues. sGCβ1-immunopositive cells were observed in the proximity of capillaries. sGCβ1-immunopositive cells had round or oval cell bodies with prominent
Fig. 2. Distribution of sGCβ1 immunoreactivity in the rat musculature. A: sGCβ1-immunopositive cells (green) have round or oval shaped cell bodies with processes (arrows) in the cross section. The immunopositive cells are located near the skeletal muscle cells with dystrophin immunoreactivity (arrowheads, red). B: sGCβ1-immunopositive cells (arrows, red) are adjacent to a capillary labeled by FITC-conjugated tomato lectin (arrowheads, green). C: Sarcolemma of the skeletal muscle cells (asterisks), including the neuromuscular junction, are negative for sGCβ1 immunoreactivity (green). Motor nerve terminals in neuromuscular junctions are labeled with the synaptophysin antibody (red, arrowheads). C shows a merged image with sGCβ1 and synaptophysin immunoreactivities and C' shows sGCβ1 immunoreactivity. D: α-Smooth muscle actin (red) immunopositive smooth muscle cells (arrows) of small blood vessels show sGCβ1 immunoreactivity (green). Endothelial cells are negative for sGCβ1 immunoreactivity (arrowheads). D shows a merged image with sGCβ1 and actin immunoreactivities and D' shows sGCβ1 immunoreactivity. Blue indicates cell nuclei stained with DAPI. Scale bar: 20 μm
Fig. 3. Distributions of sGCα1 and sGCβ1 immunoreactivities and relationships between sGC and NG2 immunoreactivities. A, B: Rabbit anti-sGCβ1 (green) and guinea pig anti-sGCβ1 (SG6) (red) immunoreactivities. In the cross section, immunoreactivities of sGCβ1 (rabbit anti-sGCβ1) (green) and SG6 (guinea pig anti-sGCβ1) (red) are observed in the same cells (arrows; yellow). The longitudinal section (B: 8 merged slices, 6.9 μm thick) indicates that sGCβ1-immunopositive cells have long cell bodies and cytoplasmic processes (arrowheads) along with skeletal muscle cells. C, D: Rabbit anti-sGCα1 (green) and guinea pig anti-sGCβ1 (SG6) (red) immunoreactivities. In the cross section, immunoreactivities of sGCα1 (green) and sGCβ1 (red) are observed in the same cells (arrows; yellow). The longitudinal section (D: 16 merged slices, 11.3 μm thick) indicate sGC-immunopositive cells have long cell bodies and cytoplasmic processes. The processes (arrowheads) extend in bilateral directions and branch off. E, F: Rabbit anti-NG2 (green) and guinea pig anti-sGCβ1 (red) immunoreactivities. In the cross section, sGCβ1-immunopositive cells (red, arrows) express NG2 immunoreactivity (green), a marker of pericytes. In the longitudinal section (F, 10 merged slices, 7.2 μm thick), cytoplasmic processes (arrowheads) of sGCβ1-immunopositive cells make contacts with neighboring cellular processes. Blue indicates cell nuclei stained by DAPI. Scale bar: 20 μm
nuclei and small amounts of surrounding cytoplasm. Cytoplasmic processes from the cell body contained several mitochondria and endoplasmic reticulum. These cells were covered with basal lamina that continued to the capillary endothelium. sGCβ1 immunoreactivity showed a high intensity in the cytoplasm of the cell body and processes. Neither capillary endothelial cells nor skeletal muscle cells did not show any sGCβ1 immunoreactivity.

Discussion

In this study, we examined the cellular distributions of sGC subunits in the skeletal musculature of rats and found the colocalization of two subunits in the same cells. sGC is a heterodimeric enzyme composed of two different subunits, α and β, known to have α1, α2, β1, and β2 subtypes (Krumenacker et al., 2004; Koesling et al., 2004); one α and one β subunit are required to form the catalytic enzyme (Krumenacker et al., 2004; Koesling, 2004). The α1β1 and α2β1 heterodimers are thought to be physiologically active toward NO (Koesling et al., 2004). Expressions of sGCα1, α2, and β1 subunits were detected in the skeletal musculature at the mRNA and protein levels (Feussner et al., 2001; Mergia et al., 2003). A study of sGCβ1 subunit-deficient mice showed that deletion of the β1 subunit gene resulted in a loss of not only the β1 subunit protein but also the α1 and α2 subunits, due to the instability of α subunits when expressed without the dimerizing partner β1 subunit (Friebe et al., 2007). Friebe et al. (2007) concluded that the β1 subunit regulated the expressions of the α-subunits and was essential for sGC function. Therefore, we primarily examined sGCβ1 subunit distributions to examine NO targets in the skeletal musculature. An immunoblot analysis using a commercial sGCβ1 antibody and newly developed sGCα1 antibody revealed the sGC distributions in the skeletal musculature. We next analyzed sGCβ1 and sGCα1 distributions using double-staining immunohistochemistry and confirmed that both subunits were distributed in the same cells in the skeletal musculature.

These immunohistochemical observations of β1 and α1 subunits of sGC revealed that the
immunopositive cells were scattered in the skeletal musculature. Antibodies for the 2 subunits labeled the same structures. The morphological characteristics of the sGC-immunopositive cells were round or oval shaped cell bodies with long, thin processes that ran parallel to the long axis of the skeletal muscle fibers. The results of double-staining immunofluorescence microscopy with sGCβ1 and dystrophin indicated that sGC-immunopositive cells were among the muscle cells. Using tomato lectin, a marker of endothelium (Morikawa et al., 2002), sGCβ1-immunopositive cells were closely situated along the sides of tomato lectin-labeled capillaries. Electron microscopic findings also supported these immunofluorescence observations. Using NG2, a marker of pericytes (Ozerdem et al., 2001; Armulik et al., 2005), sGCβ1 immunoreactivity was observed in NG2-immunopositive cells. Previous electron microscope analyses have revealed the morphological characteristics of capillary pericytes. Scanning electron microscopic studies showed that pericytes extended long, thin primary bilateral processes along the axes of capillaries in rat cardiac muscle and ciliary processes (Fujiwara et al., 1999; Higuchi et al., 2000). These morphological characteristics were similar to those of the sGCβ1-immunopositive cells in this study. From these observations, we concluded that the sGCα1 and β1-immunopositive cells were pericytes around the microvasculature.

Previous studies have reported on sGC distributions in the skeletal musculature. Feussner et al. (2001) found that the mammalian musculature contained the α1, β1, and β2 subunits of sGC in the sarcolemma region. Buchwalow et al. (2005) reported that sGC immunoreactivity in the skeletal musculature of rat quadriceps was detected not only in the sarcolemma but also in subcellular compartments such as the sarcoplasmic reticulum and mitochondria. Schoser et al. (2001) found that sGCα2 was in the sarcolemma region and that sGCα1/β1 subunits were in the neuromuscular junction of the human skeletal musculature. In this study, the three sGC antibodies used did not label sarcolemma or neuromuscular junctions in the rat skeletal musculature. Although the discrepancy in the immunohistochemical results between our data and the previous data is not clear, we sometimes observed non-specific immunofluorescence or autofluorescence in the sarcolemma or cytoplasm of skeletal muscle cells. Our study showed that sGCβ1 immunoreactivity was only observed around the tomato lectin-labelled microvessels. Moreover, we attempted double-immunostaining with NG2—a marker of pericytes—and sGCβ1 and confirmed that sGCβ1 immunopositive cells were pericytes. We also observed sGCβ1 immunoreactivity around large diameter vessels and found co-localization with α-smooth muscle actin immunoreactivity. These observations that the smooth muscle cells of the blood vessels contain sGC immunoreactivity agree with Schoser’s results that the artery wall is stained with a sGCα1/β1 subunit antibody.

Our results demonstrate the expressions of the α1 and β1 sGC subunits in pericytes and suggest that pericytes in the skeletal musculature are targets of NO because sGC is considered to be a functional receptor of NO. In retinal blood vessels, NO inhibited calcium channels in pericytes-containing microvessels and subsequently decreased the contractile tone of pericytes (Sakagami et al., 2001). In the human and guinea pig urinary bladders and the proximal urethras, immunoreactivity to cGMP was observed in the smooth musculature of blood vessels and pericytes (Smet et al., 1996). Joyce et al. (1984) reported that cGMP-dependent protein kinase was distributed in the microvascular pericytes and vascular smooth muscle cells in rat tissues such as the heart, diaphragm, intestine, and mesentery. These results suggest that pericytes are regulated by response to NO and modulate microvascular blood flow. In the skeletal musculature, NO is produced by the enzymatic actions of not only eNOS but also the nNOS that is abundantly expressed in skeletal muscle cells (Stamler and Meissner, 2001). NO produced from nNOS in the skeletal musculature during exercise has been shown to regulate local blood flow by diffusing from skeletal muscle cells to the microvessels (Thomas et al., 2003; Kobayashin et al., 2008). Pericytes in the skeletal musculature are involved with blood flow in the microvasculature via NO-sGC signaling.

It has been suggested that pericytes can differentiate into some types of mesenchymal cells—such as vascular smooth muscle cells—in conjunction with vessel growth and remodeling (Armulik et al., 2005). In addition, pericytes may give rise to fibroblasts, osteoblasts, chondrocytes and adipocytes (Armulik A, 2005). Dellavalle et al. (2007) also described pericytes as representing a myogenic precursor, resident in the adult human skeletal musculature, with a myogenic potency similar to, but phenotypically distinct from, satellite cells. They showed that isolated pericytes that expressed NG2 progressively differentiated into myogenic precursors. These pericyte-derived myogenic precursors colonized in the musculature of mice with muscular dystrophy and differentiated into dystrophin-expressing skeletal muscle cells. On the other hand, satellite cells in the skeletal musculature are the best known myogenic precursor cells and are regulated by NO during their activation (Anderson, 2000; Wozniak and Anderson, 2007). To date,
there have been no findings regarding the regulation of pericytes’ differentiation by NO. Thus, the localization of sGC in pericytes suggests the functional significance of NO for their differentiation.

References


