

Fig. 2. Serum CK value in acute hindlimb ischemia. Open bar, control group; closed bar, iNOS-knockout (iNOS-KO) group; hatched bar, aminoguanidine-treated group (Wild + AG). The CK values in the iNOS-KO group and Wild + AG group were significantly higher than that in the control (Wild) group (\* $p < 0.05$ ).

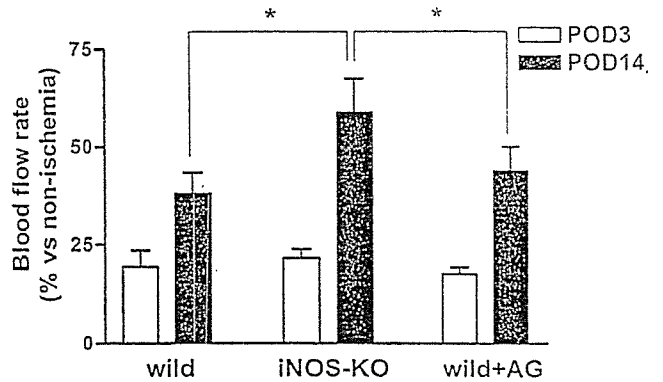


Fig. 4. Blood flow rate in non-contact laser-Doppler flowmetry. Open bar, blood flow ratio on day 3. Closed bar, blood flow ratio on day 14. The blood flow ratio on day 14 in the iNOS-KO group was significantly higher than in the Wild group or Wild + AG group (\* $p < 0.05$ ).

the Wild group or the Wild + AG group ( $0.12 \pm 0.01$  and  $0.15 \pm 0.02$ , respectively) ( $p < 0.05$ ) (Figure 5).

*FITC angiography*—The presence of fine vascular networks in the iNOS-KO group (Fig. 6B) implies that marked angiogenesis had occurred. There was a distinct difference in induction of vascular networks between the iNOS-KO group and the Wild and Wild + AG groups (Figure 6 A,B,C).

Discussion

In this experiment, ischemic injury was severe, but angiogenesis was markedly greater in the iNOS-KO group than in the Wild + AG or Wild group. The results indicate that NO generated by iNOS inhibited acute ischemic injury, but reduced angiogenesis in the chronic stage of ischemia.

Of the three NO synthase isoforms, nNOS is mainly localized in the central nervous system, postsynaptic density (PSD), and muscular sarcolemma (muscle fiber myelin) and participates in neural transmission [39-41]. iNOS is usually not expressed, but is induced in vascular smooth muscle cells and macrophages *via* cytokine stimulation during sepsis or ischemia with or without reperfusion, and produces large quantities of NO [42]. eNOS is mainly localized in vascular endothelial cells and produces NO continuously in response to shear stress, playing important roles in platelet aggregation and vasodilation [12, 17]. So, it appears reasonable that NO inhibits acute ischemic injury. In contrast, many studies have shown that NO production by iNOS aggravates injury in the hindlimb ischemia model. Nevertheless, we found that iNOS reduced ischemic injury in the acute stage in the present experiment. A key difference between our study and the others is the presence or absence of reperfusion following the ischemic period. We have

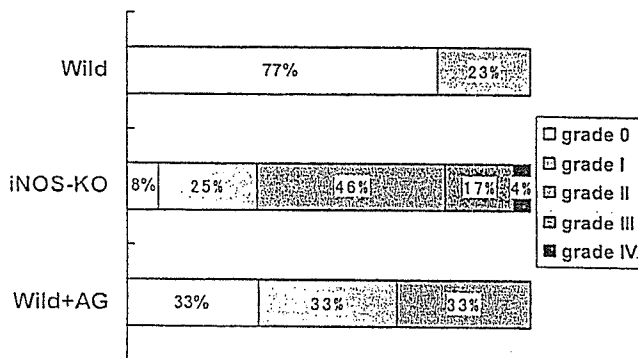


Fig. 3. Ischemic score in acute hindlimb ischemia. Open column, grade 0; dotted column, grade I; vertically lined column, grade II; hatched column, grade III; cross lined column, grade IV.

blood flow at the ischemic lesion was significantly reduced in all three groups on day 3 after surgery. However, at post-operative day 14, it was significantly higher in the iNOS-KO group ( $58.7 \pm 8.7\%$ ) than in the Wild group ( $38.1 \pm 5.2\%$ ) or the Wild + AG group ( $43.5 \pm 6.4\%$ ) (Figure 4).

*Sequential microangiography in vivo*—No vessels were apparent in hind limb angiography on day 0, and fine vessels were barely visible on day 14 in the Wild group (Figure 5 A and B). In contrast, many vessels were supplying the hind-limb on the injured side on day 14 in the iNOS-KO group. The angiogenic score on day 14 was significantly increased in the iNOS-KO group ( $0.29 \pm 0.02$ ) compared with that in

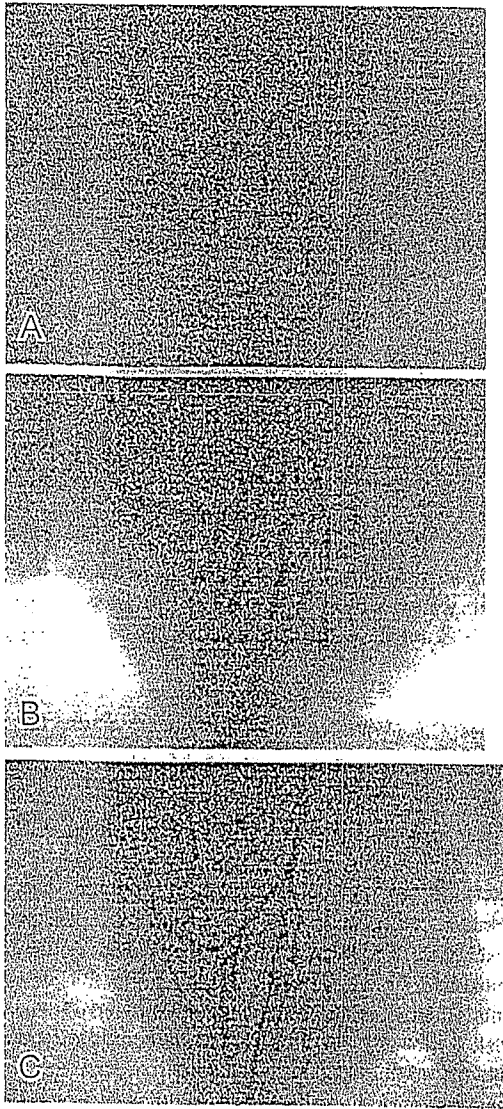


Fig. 5. Representative microangiograms. A : angiogram taken on day 0 in an animal of the Wild group ; B : angiogram taken on day 14 in an animal of the Wild group ; C : angiogram taken on day 14 in an animal of the iNOS-KO group.

already reported that superoxide ( $O_2^{\cdot-}$ ) is produced in ischemic tissue at the time of reperfusion, and reacts with NO to form peroxynitrite [43]. Peroxynitrite is a potent oxidant that directly oxidizes sulfhydryl groups at a 1000-fold greater rate than hydrogen peroxide. It inhibits the function of various enzymes, including components of the mitochondrial electron transport chain. In our experiment, we examined ischemia without reperfusion, so that  $O_2^{\cdot-}$  (and hence peroxynitrite) would not be produced, and only NO was present.

A second difference from previous experiments is that we used mice treated with iNOS inhibitor in the acute stage, as

well as iNOS knockout mice, to examine the effect of iNOS [44]. It is noteworthy that one study in which iNOS knockout mice were used and reperfusion was not performed (similar to our protocol) found that injury was severe and angiogenesis in the chronic stage was augmented [45]. This is consistent with our results, and indicates that reperfusion plays a critical role in the outcome [46].

As the ischemic signal in the acute phase is a critical factor inducing angiogenesis [34], and angiogenesis increases in proportion to the degree of ischemia [35, 36], angiogenesis has to be compared among groups with comparable severity of acute ischemic injury. We therefore selected animals with grade I ischemic score in all cases for comparison among groups. Aminoguanidine was administered for only three days in the Wild + AG group in order to allow iNOS to function in the chronic stage. At corresponding levels of acute ischemia, angiogenesis in the chronic stage was obviously enhanced in the iNOS-KO group in comparison with the Wild + AG group, i.e., angiogenesis in the chronic stage was inhibited by the function of iNOS.

Many reports indicate that iNOS enhances angiogenesis in various neoplastic disease models [44, 47–49]. However, factors secreted by the cancer cells may play important roles in these models. It is important to note that our results showing a biphasic action of iNOS depended on the use of both an acutely iNOS inhibitor-treated wild-type group and an iNOS knockout group in an ischemic model. The mechanism underlying the inhibitory action of NO appears to be down-regulation of the VEGF receptor [50]. Possible compensatory roles of eNOS and nNOS in iNOS knockout mice have been ruled out by a previous study, in which their expression was shown to remain unchanged [51].

In summary, we have shown that iNOS reduces acute ischemia, but inhibits angiogenesis in a hindlimb ischemia model. Thus we suggest to use iNOS inducer or agents to increase NO production such as arginine with acute phase and supplement iNOS inhibitor in chronic stage. However these remained to be examined prior to clinical trial.

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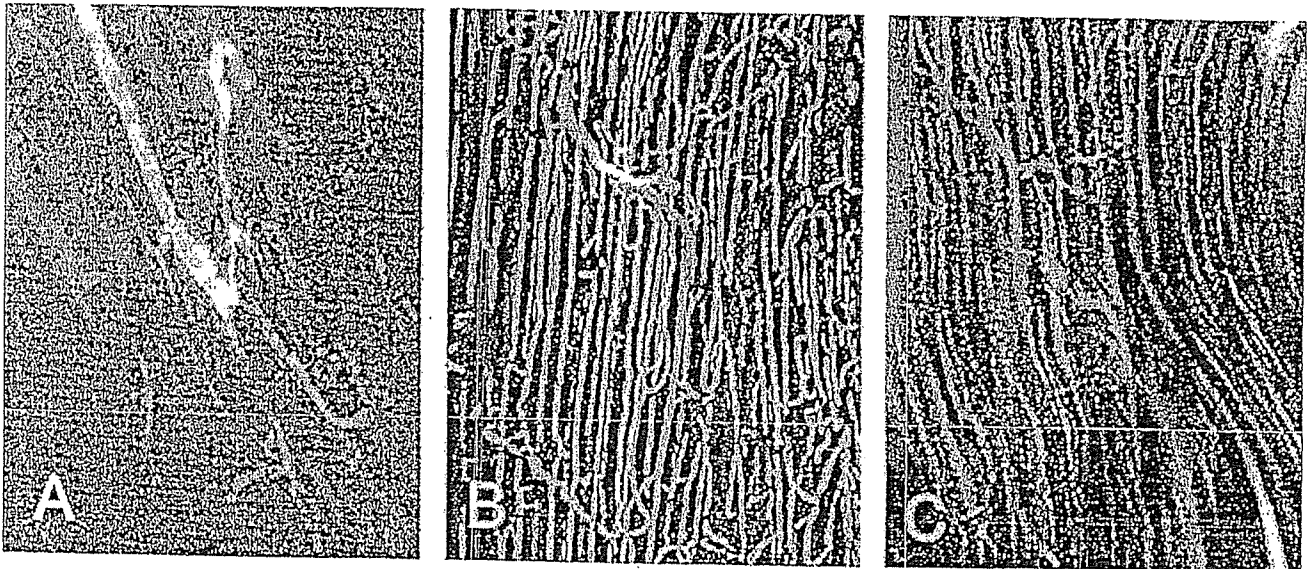


Fig. 6. FITC angiography. FITC angiograms were evaluated on day 14. A: in an animal of the Wild group; B: in an animal of the iNOS-KO group; C: in an animal of the Wild + AG group. Clear angiogenesis was visualized in the iNOS-KO group compared with the control group and aminoguanidine-treated group.

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## Effect of sustained limb ischemia on norepinephrine release from skeletal muscle sympathetic nerve endings

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### Abstract

Acute ischemia has been reported to impair sympathetic outflow distal to the ischemic area in various organs, whereas relatively little is known about this phenomenon in skeletal muscle. We examined how acute ischemia affects norepinephrine (NE) release at skeletal muscle sympathetic nerve endings. We implanted a dialysis probe into the adductor muscle in anesthetized rabbits and measured dialysate NE levels as an index of skeletal muscle interstitial NE levels. Regional ischemia was introduced by microsphere injection and ligation of the common iliac artery. The time courses of dialysate NE levels were examined during prolonged ischemia. Ischemia induced a decrease in the dialysate NE level (from  $19 \pm 4$  to  $2.0 \pm 0$  pg/ml, mean  $\pm$  S.E.), and then a progressive increase in the dialysate NE level. The increment in the dialysate NE level was examined with local administration of desipramine (DMI, a membrane NE transport inhibitor),  $\omega$ -conotoxin GVIA (CTX, an N-type  $\text{Ca}^{2+}$  channel blocker), or TMB-8 (an intracellular  $\text{Ca}^{2+}$  antagonist). At 4 h ischemia, the increment in the dialysate NE level (vehicle group,  $143 \pm 30$  pg/ml) was suppressed by TMB-8 ( $25 \pm 5$  pg/ml) but not by DMI ( $128 \pm 10$  pg/ml) or CTX ( $122 \pm 18$  pg/ml). At 6 h ischemia, the increment in the dialysate NE level was not suppressed by the pretreatment. Ischemia induced biphasic responses in the skeletal muscle. Initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE release function, while in the later phase, the skeletal muscle ischemia-induced NE release was partly attributable to exocytosis via intracellular  $\text{Ca}^{2+}$  overload rather than opening of calcium channels or carrier mediated outward transport of NE.  
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**Keywords:** Catecholamine; Interstitial space; Microdialysis; Rabbit; Striate muscle

### 1. Introduction

Acute ischemia has been reported to be associated with impairment of the sympathetic tract (Schömig et al., 1984; Toyohara et al., 1986; Fujii et al., 2003). A well-known example is myocardial ischemia associated with impairment of the regional cardiac sympathetic nerve endings (Schömig et al., 1984; Ciuffo et al., 1985). Outward norepinephrine (NE) transport through uptake<sub>1</sub> carrier has been proposed as one of the main mechanisms responsible for ischemia-induced NE efflux from sympathetic nerve endings (Schömig et al., 1984; Akiyama and Yamazaki, 2001). However, little is known about the sympathetic impairment evoked by skeletal muscle ischemia. Histochemical and electrophysiological studies

(Barker and Saito, 1981; Hill et al., 1996) have identified sympathetic innervation in skeletal muscle, which exerted actions on the regulation of regional blood flow and glucose metabolism (Thompson and Mohrman, 1983; Fagius and Berne, 1994). During and after exercise, muscle sympathetic nerve activity has been reported to be modulated by ischemia-induced metaboreceptor stimulation (Comett et al., 2000; Cui et al., 2001). Furthermore, skeletal muscle may be exposed to prolonged severe ischemia (Welsh and Lindinger, 1993). Severe skeletal muscle ischemia occurs with trauma, vascular diseases, and compartment syndrome. It is so far unknown whether severe muscle ischemia induces excessive NE release from muscle sympathetic nerve endings.

In view of energy metabolism, cardiac ischemia is characterized by rapid deterioration of cardiac function, which has been linked to a fall in intracellular pH, increased levels of inorganic phosphate and reduction in free energy changes of ATP-hydrolysis (Mair, 1999). In contrast to cardiac muscle,

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energy requirements in skeletal muscle are dependent on exercise and are reduced in the resting state since only resting tone is maintained (Idström et al., 1990; Lindsay et al., 1990). Typically, prolonged skeletal muscle ischemia imposes a metabolic stress that results in a depletion of glycogen, high-energy phosphagen, and adenine nucleotides (Welsh and Lindinger, 1993). Thus, a differential time course of energy metabolism occurs in the skeletal muscle and cardiac myocardium. No studies have systematically characterized the impairment of sympathetic nerves in the skeletal muscle ischemia.

Recently, we reported that microdialysis technique with high-performance liquid chromatography is a sensitive and versatile method for monitoring interstitial NE concentrations in myocardial ischemic regions (Akiyama et al., 1991, 1993). Moreover, we applied microdialysis technique to skeletal muscle and have reported that skeletal muscle dialysate NE serves as an index of muscle sympathetic nerve activity (Tokunaga et al., 2003a). Using this method, we investigated how acute skeletal muscle ischemia affects NE release from skeletal muscle sympathetic nerve endings and the mechanism of skeletal muscle ischemia-induced NE release with regional pharmacological intervention.

## 2. Methods

### 2.1. Animal model

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Forty-two male Japanese white rabbits weighing 2.2–3.8 kg were used for the model of skeletal muscle ischemia. The animals were anesthetized with pentobarbital sodium (30–35 mg/kg) and ventilated with room air mixed with oxygen. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). Body temperature was maintained with a heated pad and lamp. An electrocardiogram, heart rate (HR), and mean arterial blood pressure (MAP) were simultaneously monitored with a data recorder. After a longitudinal skin incision was made in the left groin, the dialysis probes were implanted in the left adductor muscle with a fine guiding needle.

### 2.2. Dialysis technique and NE measurements

With the dialysis technique, dialysate NE levels were measured as an index of skeletal muscle interstitial NE levels. For skeletal muscle dialysis, we designed a transverse dialysis probe. The dialysis fiber (13 mm length, 0.31 mm o.d. and 0.2 mm i.d.; PAN-1200, 50,000 molecular mass cut-off, Asahi Chemical, Tokyo, Japan) was glued at both ends into a polyethylene tube (25 cm length, 0.5 mm o.d. and 0.2 mm i.d.) (Akiyama et al., 1991). The dialysis probe was perfused with Ringer solution using a microinjection pump (CMA 102, Carmergie Medicin, Stockholm, Sweden). Similar to previous studies (Tokunaga et al., 2003a, 2003b), we chose a perfusion speed of 10  $\mu$ l/min for skeletal muscle. Sampling periods were set at 15 min for skeletal muscle. Dialysate NE levels were measured by high-performance liquid chromatography with electrochemical detection (ECD-300, Eicom, Kyoto, Japan) after removing interfering compounds in the dialysate by an alumina procedure (Anton and Sayer, 1962; Akiyama et al., 1991). Dialysate dihydroxyphenylglycol (DHPG) levels were measured by separate high-performance liquid chromatography with electrochemical detection (Akiyama and Yamazaki, 2001).

### 2.3. Experimental protocols

Acute skeletal muscle ischemia was induced by injection of non-radioactive iodine-labeled microspheres (15  $\mu$ m in diameter,  $3 \times 10^7$ /kg, Sekisui Plastic,

Osaka, Japan) through the left common iliac artery, as previously described (Tanaka et al., 2000). After the injection of microspheres, the common iliac artery was ligated.

#### 2.3.1. Protocol 1: time courses of dialysate NE levels during acute ischemia

To examine the time courses of dialysate NE levels during acute skeletal muscle ischemia, we measured dialysate NE levels over 60-min periods of skeletal muscle ischemia ( $n = 6$ ). We collected four consecutive 15-min dialysate samples. Furthermore, we measured dialysate NE samples over a period of 6 h of skeletal muscle ischemia with 2 h interval in separate rabbits. To examine intraneuronal NE kinetics in the skeletal muscle, the measurement of dialysate DHPG level was added during 6 h of skeletal muscle ischemia ( $n = 6$ ).

#### 2.3.2. Protocol 2: involvement of NE uptake, transport, $Ca^{2+}$ channels and cytosol $Ca^{2+}$ in dialysate NE levels during acute ischemia

To examine the mechanism underlying the increment of NE release during the prolonged ischemia, dialysate NE levels were measured with regional pharmacological intervention. Neurotransmitter release from sympathetic nerve endings can be caused by a variety of different mechanisms (Schömig et al., 1987; Kawada et al., 2000; Akiyama and Yamazaki, 2001). In the present studies, we examined the roles of membrane NE transport, N-type  $Ca^{2+}$  channels and cytosol  $Ca^{2+}$  in the time courses of dialysate NE levels during prolonged ischemia. To examine the involvement of membrane NE transport in the ischemia-induced NE release, we locally administered an uptake, carrier blocker, desipramine (100  $\mu$ M) through a dialysis probe and observed the responses of dialysate NE (Akiyama and Yamazaki, 2001) ( $n = 6$ ). The same protocol was performed with addition of a voltage-dependent N-type  $Ca^{2+}$  channel blocker,  $\omega$ -conotoxin GVIA (10  $\mu$ M) ( $n = 6$ ) or intracellular  $Ca^{2+}$  antagonist, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, 1 mM) ( $n = 6$ ) through a dialysis probe. From data on protocol 1, we observed increases in dialysate NE levels after 2 h of skeletal muscle ischemia. Therefore, the time course of dialysate NE for skeletal muscle ischemia was examined over a period of 6 h with a 2 h-interval ( $n = 6$ ). The effectiveness of  $\omega$ -conotoxin GVIA (10  $\mu$ M) ( $n = 6$ ) or TMB-8 (1 mM) ( $n = 6$ ) was tested before the experiment in separate rabbits. We administered high potassium (KCl, 100 mM) locally through the dialysis probe, and the dialysate NE response was obtained in the presence and absence of  $\omega$ -conotoxin GVIA or TMB-8. High-K increased dialysate NE from  $11.7 \pm 2.8$  to  $84.7 \pm 20.8$  pg/ml ( $n = 6$ ). This KCl-induced increment in dialysate NE was attenuated by the addition of  $\omega$ -conotoxin GVIA or TMB-8 (Fig. 1).

#### 2.3.3. Protocol 3: time courses of dialysate lactate levels during the hind limb ischemia

To confirm whether this perturbation induces tissue ischemia, we examined the time course of dialysate lactate levels as an index of tissue ischemia. The dialysate lactate levels were measured by kinetic enzymatic analysis with CMA 600 (Carnegie Medicin). In the skeletal muscle ischemia, four consecutive 15-

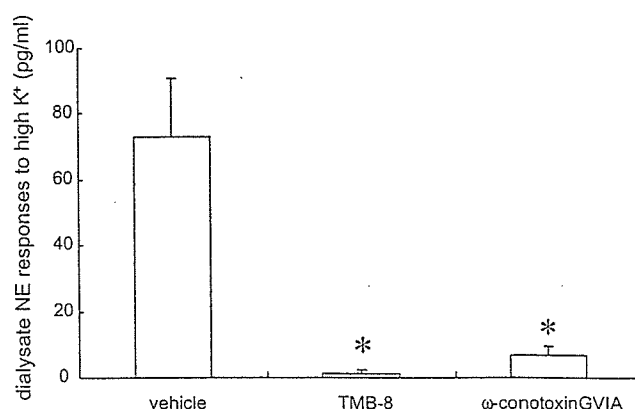


Fig. 1. Effects of pharmacological intervention on dialysate norepinephrine (NE) responses to high K<sup>+</sup> (KCl, 100 mM). Both TMB-8 (1 mM) and  $\omega$ -conotoxin (10  $\mu$ M) suppressed dialysate responses to high K<sup>+</sup>. Values are means  $\pm$  S.E. ( $n = 6$ ).



min dialysate samples were collected during the initial 60-min and subsequently three consecutive samples were collected over a period of 6 h with a 2 h-interval ( $n = 6$ ).

#### 2.4. Statistical analysis

All data are presented as mean  $\pm$  S.E. values. Hemodynamic and dialysate data responses to acute ischemia were statistically analyzed by analysis of variance with repeated measures. When a statistically significant effect of ischemia was detected as a whole, the Dunnett's test was applied to determine which mean values differed significantly from the control level. When a statistically significant effect of the treatment was detected, Newman-Keuls test was applied to determine which treatment differed significantly from the vehicle.

### 3. Results

Table 1 summarizes changes in HR and MAP. MAP and HR increased during 6 h-hind limb ischemia. Changes in MAP at 2 h and HR at 6 h-hind limb ischemia were significant.

#### 3.1. Time courses of dialysate NE levels during short and prolonged ischemia

Skeletal muscle dialysate NE levels decreased from  $19 \pm 4$  pg/ml at control to  $9 \pm 4$  pg/ml at 30 min of ischemia and reached  $2 \pm 0$  pg/ml at 60 min of ischemia (Fig. 2). The decrease in dialysate NE level was maintained after 2 h of ischemia. Then skeletal muscle dialysate NE levels markedly increased to  $143 \pm 30$  pg/ml at 4 h of ischemia. The dialysate NE levels continued to increase progressively and reached  $289 \pm 45$  pg/ml at 6 h of ischemia. Skeletal muscle dialysate DHPG levels decreased from  $38 \pm 2$  pg/ml at control to  $5 \pm 1$  pg/ml at 2 h of ischemia and reached  $7 \pm 1$  pg/ml at 6 h of ischemia.

#### 3.2. Involvement of NE uptake, transport, $Ca^{2+}$ channels and cytosol $Ca^{2+}$ in dialysate NE levels during prolonged ischemia

Dialysate NE increases at 4 and 6 h-skeletal muscle ischemia were not suppressed by treatment with desipramine (Fig. 3). Dialysate NE increases at 4 and 6 h-skeletal muscle ischemia were not suppressed by treatment with  $\omega$ -conotoxin GVIA. Treatment with TMB-8 significantly suppressed the dialysate NE increase at 4 h-skeletal muscle ischemia. But at 6 h-skeletal muscle ischemia, there was no significant difference in dialysate NE levels among treatments.

Table 1

Changes in heart rate (HR) and mean arterial pressure (MAP) in 6 h-hindlimb ischemia

	Control	2 h	4 h	6 h
HR (beats/min)	$283 \pm 10$	$292 \pm 4$	$293 \pm 8$	$302 \pm 8^*$
MAP (mmHg)	$104 \pm 6$	$114 \pm 3^*$	$111 \pm 4$	$108 \pm 4$

Values are means  $\pm$  S.E. from six rabbits. Data were obtained during control, after 2, 4, and 6 h of hind limb ischemia.

\*  $P < 0.05$  vs. control.

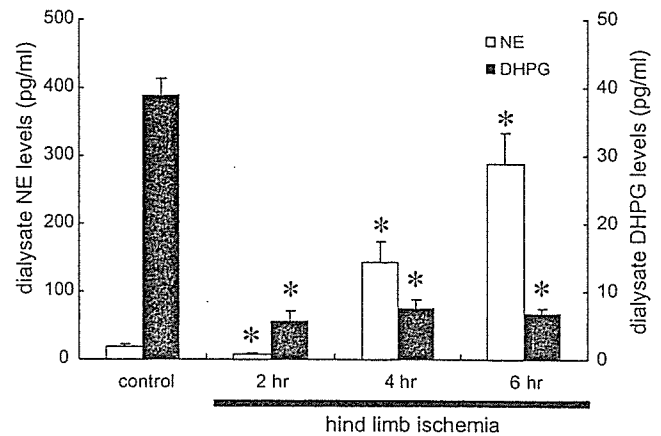
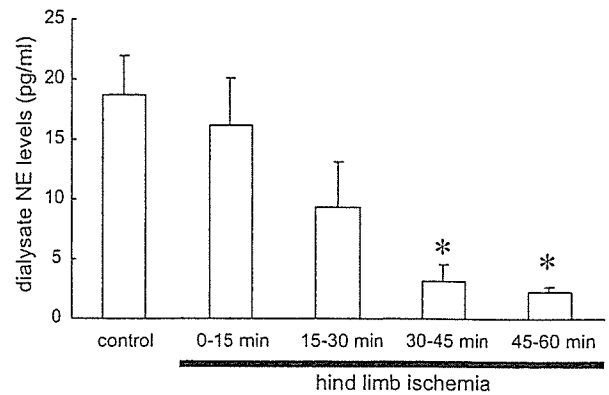


Fig. 2. (Upper panel) Time course of dialysate norepinephrine (NE) levels during 60 min-hind limb ischemia. Values are means  $\pm$  S.E. ( $n = 6$ ). \* $P < 0.05$  vs. control value. (Lower panel) Time courses of dialysate NE and dihydroxyphenylglycol (DHPG) levels during 6 h-hind limb ischemia. Values are means  $\pm$  S.E. ( $n = 6$ ). \* $P < 0.05$  vs. control value.

#### 3.3. Time course of dialysate lactate levels during hind limb ischemia

Skeletal muscle dialysate lactate levels increased from  $0.6 \pm 0.07$  nmol/l at control to  $1.73 \pm 0.17$  nmol/l at 45–60 min

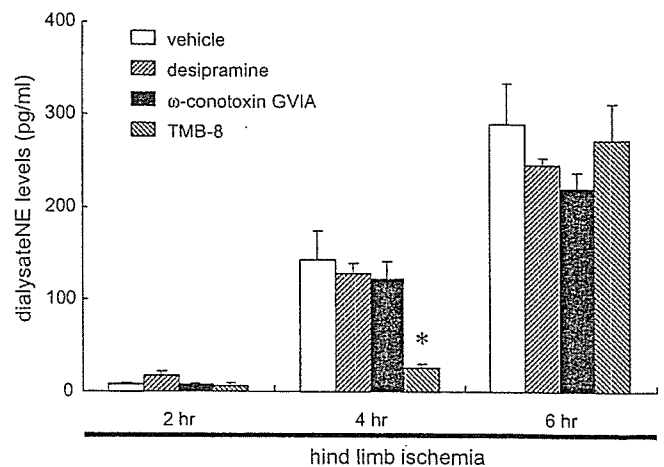


Fig. 3. Effects of pharmacological intervention on dialysate norepinephrine (NE) levels evoked by 6 h-hind limb ischemia. Desipramine (100  $\mu$ M),  $\omega$ -conotoxin (10  $\mu$ M), or TMB-8 (1 mM) was locally administered through the probe. Values are means  $\pm$  S.E. ( $n = 6$ ). \* $P < 0.05$  vs. concurrent value of vehicle group.



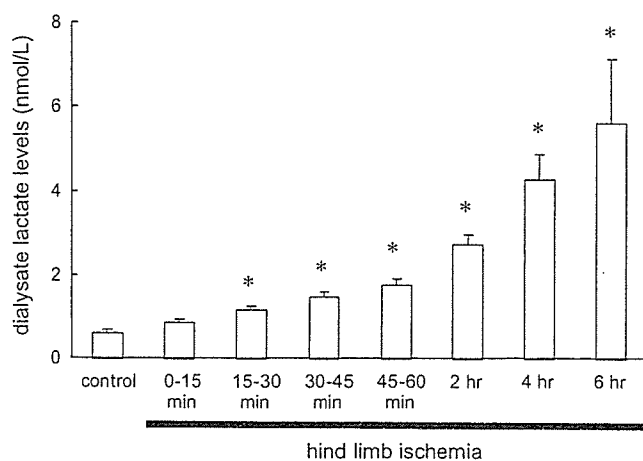


Fig. 4. Time course of dialysate lactate levels during 6 h-hind limb ischemia. Values are means  $\pm$  S.E. ( $n = 6$ ). \* $P < 0.05$  vs. control value.

of ischemia (Fig. 4). These step-wise increases were continued for 6 h of the hind limb ischemia.

#### 4. Discussion

Using dialysis techniques in the *in vivo* rabbit skeletal muscle, we examined interstitial levels of NE in the control and ischemic period, and observed the biphasic response of dialysate NE in ischemic skeletal muscle. Ischemia induced an initial reduction followed by a progressive increment in dialysate NE levels. Here we discuss changes in interstitial NE and possible mechanisms underlying sympathetic nerve impairment.

Within 2 h of acute skeletal muscle ischemia, unlike acute myocardial ischemia, skeletal muscle interstitial NE levels continued to decline progressively, decreasing to one-tenth of control at 60 min of ischemia. A previous study demonstrated that skeletal muscle ischemia modulated the baroreflex control of regional muscle sympathetic activity (Cornett et al., 2000). At 75 min of acute skeletal muscle ischemia, hemodynamic responses to carotid occlusion were preserved while the interstitial NE response to carotid occlusion was blunted in the ischemic region (Tokunaga et al., 2003b). These results indicate that the systemic response to baroreflex remained intact while the skeletal muscle sympathetic response was impaired in ischemic regions. Earlier studies reported that acute limb ischemia reduced the conduction of motor nerves such as sciatic nerve (Fern and Harrison, 1994), and induced axonal degeneration histologically (Makitie and Teravainen, 1977; Nukada and Dyck, 1987). Axonal conduction in the ischemic muscle sympathetic nerve may be impaired as well as in sensory and motor nerves. In addition to diminished axonal conductance, the interstitial NE response to high  $K^+$  but not tyramine was suppressed during the 75 min of acute skeletal muscle ischemia, although NE content at muscle sympathetic nerve endings was preserved during the ischemia (Tokunaga et al., 2003b). This result indicates that exocytotic NE releasing function in muscle sympathetic nerve endings might be suppressed during 75 min of acute skeletal muscle ischemia.

Therefore, initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE releasing function.

After 2 h of acute skeletal muscle ischemia, skeletal muscle interstitial NE levels significantly increased and finally reached 20-fold that of control. This amount of NE release is higher than that evoked by baroreflex or high  $K^+$ . This level is similar to that evoked by the  $Na^+-K^+$  ATPase inhibitor, ouabain (Tokunaga et al., 2003a). The amount of NE release evoked by ischemia may be dependent on the density of sympathetic innervation. Dispersed organ systems such as skeletal muscle have a thin and diffuse sympathetic innervation. This is the first report to describe that marked NE release is induced from muscle sympathetic nerve endings in the ischemic region after 2 h of skeletal muscle ischemia. Numerous histological changes of skeletal muscle have been reported after ischemia and reperfusion injury (Patterson and Klenerman, 1979; Turchányi et al., 2005). However, there is no histochemical evidence of the impaired sympathetic nerves in the skeletal muscle ischemia.

In the case of skeletal muscle ischemia,  $\omega$ -conotoxin GVIA did not suppress NE efflux. N-type  $Ca^{2+}$  channels are not involved in this NE efflux. Desipramine did not alter NE efflux during skeletal muscle ischemia. Desipramine inhibits carrier-mediated NE transport in both directions. Considering that desipramine did not alter interstitial NE levels, the amounts of NE release and uptake via normal transport can be surmised to be negligible. Second, the increase in skeletal muscle interstitial NE levels was not associated with an increase in skeletal muscle interstitial DHPG levels, indicating that skeletal ischemia fails to induce axoplasmic NE elevation via alterations in monoamine activity, NE mobilization from stored vesicle, and NE uptake. Further, desipramine did not suppress NE efflux. These results exclude the possibility that marked increases in skeletal muscle interstitial NE could be due to carrier-mediated outward transport of NE for removal of elevated axoplasmic NE concentration. The membrane NE transporter exists in the skeletal muscle sympathetic nerve endings (Cabassi et al., 2001; Tokunaga et al., 2003a), but was not involved in outward transport of NE. Thus, we consider that a  $\omega$ -conotoxin GVIA insensitive and desipramine-resistant NE release mechanism exists after 2 h of acute skeletal muscle ischemia.

TMB-8 significantly suppressed the marked NE release at 4 h of skeletal muscle ischemia. TMB-8 is well known to inhibit  $Ca^{2+}$  release from intracellular  $Ca^{2+}$  stores. TMB-8 inhibits caffeine-induced catecholamine release from perfused adrenal gland in the absence of extracellular  $Ca^{2+}$  (Yamada et al., 1988). Studies using chromaffin cells, brain slices and synaptosomes have suggested that metabolic inhibition induces intracellular  $Ca^{2+}$  overload (Milusheva et al., 1992), and a rise in the intracellular  $Ca^{2+}$  causes exocytotic catecholamine release without membrane depolarization (Dry et al., 1991; Du et al., 1997). Moreover, an *in vitro* study with adrenergic nerves of guinea-pig vas deferens suggested that  $Ca^{2+}$  release from intracellular  $Ca^{2+}$  stores is to some extent involved in the NE release evoked by elevation of intracellular  $Na^+$  (Katsuragi et al., 1994). Under energy-depleted conditions,  $Ca^{2+}$  overload

in synaptosomes of noradrenergic neurons from the brain is an important mechanism for the enhanced release of neurotransmitter, with a reversal of  $\text{Na}^+$ – $\text{Ca}^{2+}$  exchange possibly the key pathway leading to intraneuronal  $\text{Ca}^{2+}$  overload (Du et al., 1997). We consider that  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores is partly involved in the NE release at 4 h of skeletal muscle ischemia.

At 6 h of skeletal ischemia, increment in dialysate NE level was not suppressed by the pretreatments. This result suggests that another mechanism may be involved in NE release, which is insensitive to desipramine,  $\omega$ -conotoxin GVIA, and TMB-8. Alternatively, the NE release may occur with development of irreversible membrane damage and can no longer be inhibited by pharmacological interventions. Future work should concentrate on these aspects of NE release during the later period.

#### 4.1. Methodological considerations

The limitation of this experiment is related to the methodology and the duration of the hind limb ischemia. In a variety of these experimental models for organ ischemia, we chose microsphere injection and iliac artery occlusion for the short and prolonged hind limb ischemia model. A preliminary experiment indicated that common iliac artery occlusion did not yield severe ischemia or muscle necrosis in a chronic ischemic model because collateral flow prevents skeletal muscle ischemia. The combination of artery occlusion and injection of microsphere was used for the hind limb ischemic model. In the hind limb ischemia, however, we did not measure skeletal muscle blood flow. To confirm whether this perturbation induced reduction of blood flow and tissue ischemia, we measured dialysate lactate levels in skeletal muscle as an index of tissue ischemia. This perturbation induced increases in dialysate lactate levels. In the present study, dialysate NE responses were examined in prolonged 6 h ischemia. Temporal changes in MAP and HR appeared but sustained significant hemodynamic changes were not observed. This duration was referred to the experiments on the tourniquet application and release time (Sapega et al., 1985; Mitrev et al., 1996). Four to 6 h of ischemic periods has been thought to produce extensive and reversible damage of skeletal muscle. Therefore, data on pharmacological intervention were obtained within 6 h of skeletal muscle ischemia.

Ischemia induced biphasic NE responses in the skeletal muscle. Initial reduction of NE release may be mediated by an impairment of axonal conduction and/or NE releasing function, while in the later phase, the skeletal muscle ischemia-induced NE release was partly attributable to exocytosis via intracellular  $\text{Ca}^{2+}$  overload rather than opening of calcium channels or carrier mediated outward transport of NE.

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# Endophilin BAR domain drives membrane curvature by two newly identified structure-based mechanisms

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The crescent-shaped BAR (Bin/Amphiphysin/Rvs-homology) domain dimer is a versatile protein module that senses and generates positive membrane curvature. The BAR domain dimer of human endophilin-A1, solved at 3.1 Å, has a unique structure consisting of a pair of helix-loop appendages sprouting out from the crescent. The appendage's short helices form a hydrophobic ridge, which runs across the concave surface at its center. Examining liposome binding and tubulation *in vitro* using purified BAR domain and its mutants indicated that the ridge penetrates into the membrane bilayer and enhances liposome tubulation. BAR domain-expressing cells exhibited marked plasma membrane tubulation *in vivo*. Furthermore, a swinging-arm mutant lost liposome tubulation activity yet retaining liposome binding. These data suggested that the rigid crescent dimer shape is crucial for the tubulation. We here propose that the BAR domain drives membrane curvature by coordinate action of the crescent's scaffold mechanism and the ridge's membrane insertion in addition to membrane binding via amino-terminal amphipathic helix.

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## Introduction

Membrane dynamics in a cell, such as membrane budding, tubulation, fission and fusion, is associated with changes in membrane curvature. The crystal structure of amphiphysin BAR (Bin/Amphiphysin/Rvs-homology) domain revealed an

unexpected structural identity with arfaptin2, a binding protein to Arf and Rac small GTPases (Tarricone *et al.*, 2001), and provided a common structural base for the sensing and the formation of positive curvature membrane by BAR-family proteins (Peter *et al.*, 2004).

Endophilins are cytoplasmic proteins containing an N-terminal BAR domain and a C-terminal SH3 domain, and are involved in membrane dynamics (Schuske *et al.*, 2003; Galli and Haucke, 2004; Wenk and De Camilli, 2004). There are five endophilin genes in the mammalian genomes, endophilin A1–3 and B1–2. Both A and B types are highly conserved from nematode to human. The most extensively studied one is endophilin-A1, a brain specific protein involved in clathrin-mediated synaptic vesicle endocytosis (Ringstad *et al.*, 1997, 2001). Via SH3 domain, endophilins bind to the GTPase dynamin, a membrane scissor, and the polyphosphoinositide phosphatase synaptojanin, a clathrin-uncoater (Ringstad *et al.*, 1997; de Heuvel *et al.*, 1997; Verstreken *et al.*, 2003). The BAR domain of endophilins is classified into the N-BAR subgroup characterized by a short amphipathic helical sequence preceding the consensus BAR-domain sequence (Peter *et al.*, 2004). The N-BAR domain of endophilin-A1 binds to liposomes and induces the tubulation *in vitro*, requiring the short amphipathic helical sequence (Farsad *et al.*, 2001).

The crescent-shaped BAR dimer structure implies a simple model to drive membrane curvature: the dimer may impress its positively charged concave surface on the negatively charged membrane to form a high-curvature membrane domain (Gallop and McMahon, 2005; McMahon and Gallop, 2005). This curvature-impressing or scaffold mechanism for membrane deformation is based on an assumption that the dimer behaves as a rigid body on the membrane (Zimmerberg and Kozlov, 2006). Although the essential requirement of positively charged residues on the concave surface has been suggested (McMahon and Mills, 2004; Peter *et al.*, 2004), there have been no experimental supports for the scaffold mechanism. Here, we show the requirement of the molecular rigidity of the BAR dimer for membrane curvature on the basis of structure-oriented mutational analysis.

By determining the structure of endophilin-A1 BAR domain, we found a distinction from those of the known BAR domains: a helix-loop appendage of 30 amino acids stretch is inserted into the helix 1 of the canonical BAR domain. A pair of the helices of the appendages forms a hydrophobic ridge, which runs across the center of the concave surface of the dimer. We analyzed the function of this ridge as well as the previously proposed structure, the N-terminal amphipathic helix and the crescent main body, for membrane deformation (Peter *et al.*, 2004). N-terminal amphipathic helix is essential for membrane binding. The crescent main body of the BAR dimer is required for impressing its intrinsic curvature to the membrane. The ridge contributes to deform the membrane

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presumably by penetrating into the membrane. Our results illustrate how these three components coordinate to induce membrane deformation.

## Results

### Endophilin-A1 BAR domain has a unique appendage

The structure of the BAR domain of human endophilin-A1 (amino acid 1–247, hereafter EndA1-BAR) was solved at 3.1 Å resolution by a multi-wavelength anomalous dispersion method. The structure of EndA1-BAR dimer is similar to that of amphiphysin (Peter *et al*, 2004) and arfaptin2 (Tarricone *et al*, 2001): a crescent-shaped dimer composed of a 6-helix bundle core and two 3-helix bundle arms extended from the core (Figure 1A). The whole structure of EndA1-BAR dimer can be precisely superimposed on that of amphiphysin and arfaptin (Figure 1B). All three structures show nearly identical dimer shapes. Notably, the present EndA1-BAR structure from a tetragonal crystal packing is almost completely the same as an independent crystal structure from an orthogonal crystal packing (Supplementary Figure 1; and Weissenhorn, 2005). The RMS deviations are 0.63, 0.86 and 0.80 Å for C $\alpha$  atoms in monomers A, B and dimer, respectively. The structural identity indicates that the crescent shape is stably present in solution. Consistent with previous results (Habermann, 2004; Peter *et al*, 2004), structure-based sequence alignment reveals that these three proteins are poorly conserved in amino-acid sequence including the residues possibly important for the crescent-shape formation (Supplementary Figure 2).

We find a unique structure of the EndA1-BAR, an appendage-like structure protruded from the center of the dimer (Figure 1A). The sequence alignments of the BAR-family proteins indicated that this appendage appears

unique to the endophilin-family proteins including nadrin (Habermann, 2004; Peter *et al*, 2004) and the candidates from yeasts (Supplementary Figure 2). The appendage (Q59–Q88) has an N-terminal short helix and a loop of which electron density is mostly missing (N72–G85). The pair of helices appears to stay on the main body and forms a ridge across the center of the concave dimer surface. The helix displays, on its top surface, a series of hydrophobic residues (P62, A63, A66 and M70) aligned 60° against the longitudinal axis of the dimer (Figure 1C). Other than the conserved hydrophobic amino acids of the ridge, the appendage sequences show clear distinction between endophilin-A and endophilin-B (Supplementary Figure 2). The B type endophilins show cytoplasmic localization, presumably being involved in intracellular membrane dynamics (Farsad *et al*, 2001; Modregger *et al*, 2003; Karbowski *et al*, 2004). Analyses of chimeric mutations in the appendage between EndA1-BAR and EndB1-BAR suggest that BAR domain may contribute to defining where to target, plasma membrane or intracellular organ membrane (Supplementary Figure 3).

### The appendage's penetration enhances liposome tubulation

To investigate the functional significance of the hydrophobic ridge of the endophilin-specific appendage, we first examined the effects of point mutations in this region (red residues in Figure 1C) on the liposome binding and tubulation activities *in vitro* (Figures 2A and 3). Introduction of membrane-repulsive negative charge (A66D) lost the ability to form tubes from liposomes. Hydrophilic mutations (A63S/A66S (SS) and A63S/A66S/M70Q (SSQ)) reduced the number of tubes (<1/100) and induced three-time enlargement of the tube diameter. In contrast, a bulky hydrophobic residue

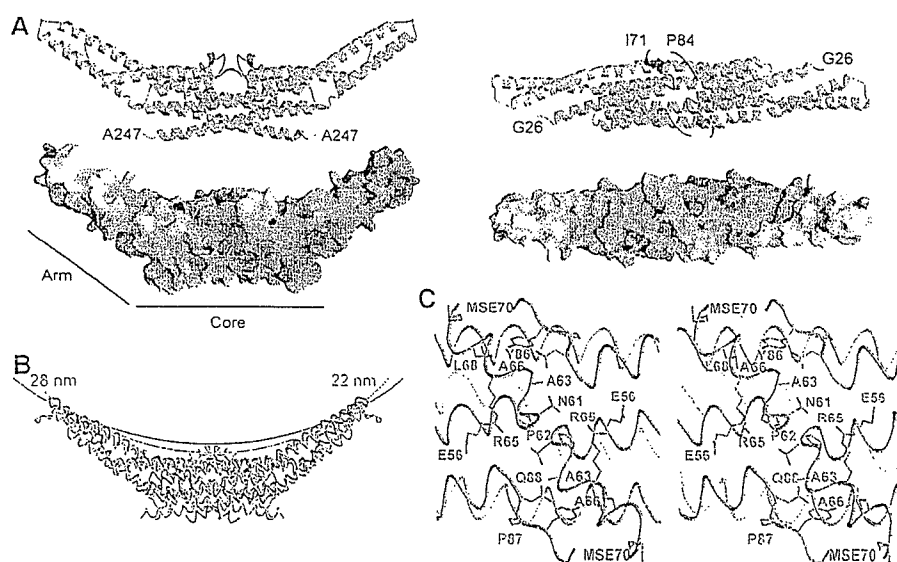


Figure 1 Structure of human endophilin-A1 BAR domain dimer. (A) Ribbon representation (a green monomer with a red appendage and a pale-blue monomer with a blue appendage) and surface electrostatic potential (red,  $-15 \text{ kTe}^{-1}$ ; blue,  $15 \text{ kTe}^{-1}$ ) of the dimer viewed from the side (left) and from the top (right). The numbered amino-acid residues are the first and the last ones in consecutive polypeptide segments determined in this model. (B) Comparison of three BAR domain structures in trace representation. Red, endophilin-A1 (PDB ID: 1X03); green, amphiphysin (1URU); blue, arfaptin2 (1I4D). The red and green arcs with indicated diameters represent curved membranes fit the concave surface of endophilin-A1 and amphiphysin, respectively. (C) Stereo view of the appendages. Side-chains of the residues forming the hydrophobic ridge and those of interacting with residues of the main body are shown.

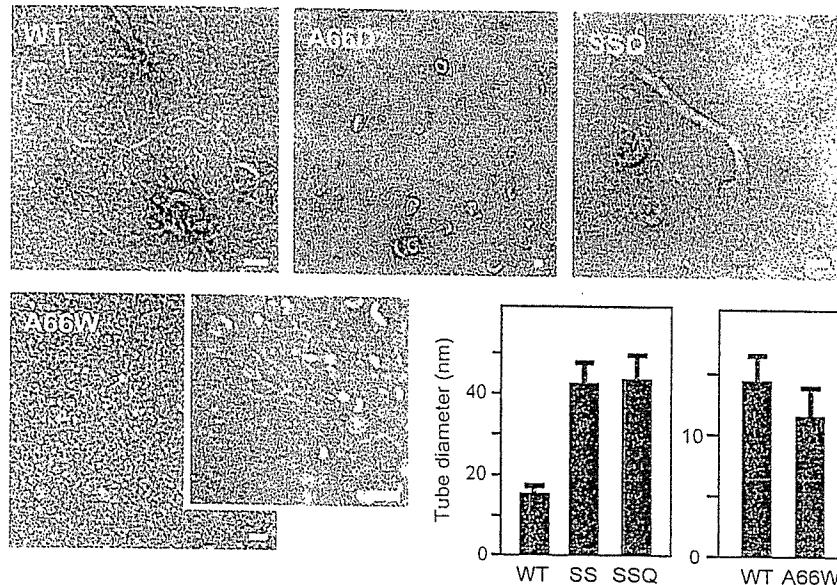


Figure 2 Liposome tubulation by endophilin-A1 BAR domains with mutations in the hydrophobic ridge. WT, 7  $\mu$ M wild-type BAR domain incubated for 10 min; A66D, 28  $\mu$ M, 10 min; SSQ, A63S/A66S/M70Q triple mutant, 28  $\mu$ M, 10 min; A66W, 1.4  $\mu$ M, 10 min (vesiculated, left panel) and 10 s (tubulated, right panel). Tubulation was not observed when incubated for longer than 1 min. Scale, 100 nm. The bar graphs show tubule diameter (mean and s.d.). SS, A63S/A66S double mutant, 28  $\mu$ M, 10 min.

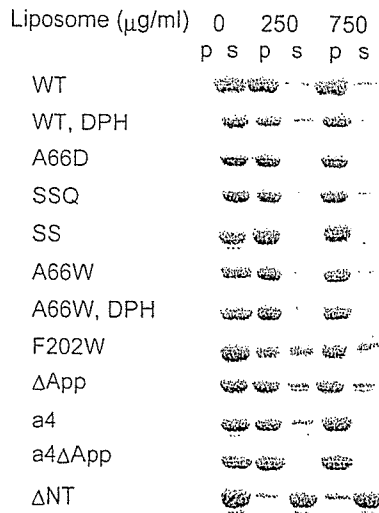


Figure 3 Liposome binding assays of endophilin-A1 BAR domain and its mutants. Protein (200  $\mu$ g/ml) was co-sedimented with liposomes (0, 250 and 750  $\mu$ g/ml). Proteins recovered from the pellet (p) and the supernatant (s) were analyzed by SDS-PAGE. The DPH-liposomes show similar binding capacity for the wild type (WT) and the A66W mutants. The liposome binding activity is slightly reduced in the F202W and the appendage-less mutants ( $\Delta$ App) and is almost lost in the helix 0 truncated mutant ( $\Delta$ NT).

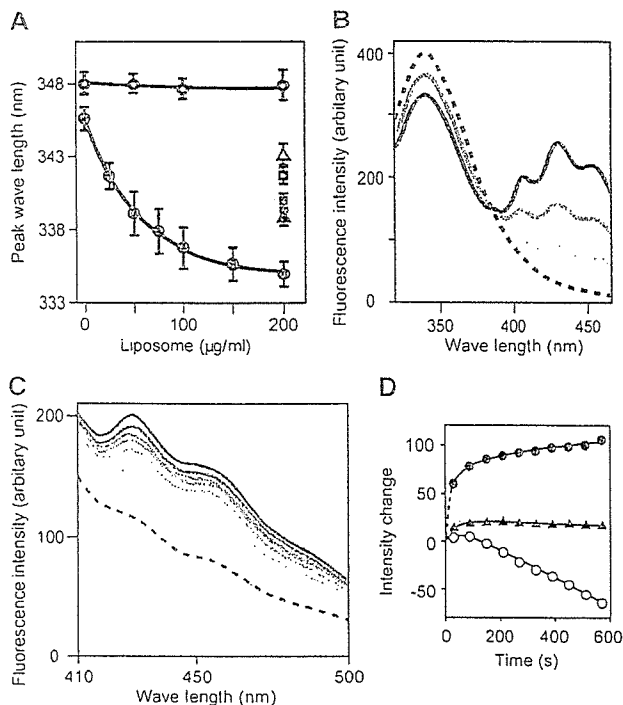
(A66W) led to extensive vesiculation and less tubulation. All these mutations did not affect the liposome binding. These results suggest an important role for the hydrophobic ridge in the membrane curvature formation but not in the membrane binding.

Although the ridge reduces the intrinsic curvature of the concave surface (red line in Figure 1B), it appears to promote the membrane curvature formation with conserved hydrophobicity. This raises the possibility that the ridge penetrates

into the membrane when the concave surface makes tight contact with the membrane. This possibility was investigated using tryptophan fluorescence, which is sensitive to hydrophobicity of the microenvironment around the indole moiety. The A66W mutant showed 10-nm blueshift of the fluorescence peak in a liposome-dose-dependent and saturable manner, while F202W, a control mutant in which Phe202 on the convex surface was mutated to Trp, did not show any shift (Figure 4A and Supplementary Figure 5). The amount of the blueshift was greater than that observed in 50% DMSO or 50% methanol, indicating that the indole moiety was in a highly hydrophobic environment.

To determine whether this blueshift was caused by the insertion of the indole moiety into the hydrophobic core of the lipid bilayer, we made fluorescence resonance energy transfer (FRET) assays using diphenyl-hexatriene (DPH) as the acceptor probe. DPH has been shown to insert specifically in the nonpolar interior of the membrane and not to alter the membrane structure and dynamics (Repáková *et al*, 2005). DPH liposomes did not affect liposome binding and tubulation (Figure 3 and Supplementary Figure 4). A66W but not F202W showed effective FRET from the 340-nm tryptophan fluorescence (donor) to the DPH fluorescence (acceptor) peaked at 430 nm (Figure 4B and C). It was not caused by changes in the fluorescence property of DPH itself possibly accompanied by tubulation/vesiculation of liposomes (Figure 4D and Supplementary Figure 6). These data suggest that the indole ring of 66W penetrates into the hydrophobic core of the membrane and that the remaining residues of the ridge, about 8 Å in height, appear to be embedded in the layer of lipid head-groups of the contacting membrane leaflet. These results confirmed that the ridge is contacting membrane and that the convex is not contacting membrane surface.

To provide further support for the membrane insertion of the ridge in the wild-type EndA1-BAR, we made a mutant



**Figure 4** Tryptophan fluorescence blueshift and FRET assays. (A) Tryptophan fluorescence emission peak when excited at 280 nm was observed in different concentration of liposome. A66W (●), F202W control mutant (○), A66W alone in 50% DMSO (▲), in 50% MeOH (■), F202W alone in 50% DMSO (△), in 50% MeOH (□), 140 µg/ml protein for all measurements. Mean and s.d. ( $N = 4-11$ ). The dose dependency is significant ( $P \ll 0.001$ ) for the A66W mutant but insignificant ( $P > 0.8$ ) for the F202W mutant (one-way ANOVA). DMSO and MeOH were used as blueshift inducer for tryptophan. (B) Dose-dependent FRET efficiency from the A66W tryptophan to DPH incorporated in liposomes was examined by the changes of fluorescence. Fluorescence spectrum of A66W (100 µg/ml) with the control liposome (200 µg/ml) excited at 280 nm (hatched). Pale to dark solid curves represent DPH:lipid weight ratios of 1:2000, 1:1000 and 1:500 in the same condition. (C) Time-dependent increase in the FRET efficiency from either A66W (pale to dark solid lines, from 30 to 570 s) or F202W tryptophan (pale and dark hatched lines, at 30 and 570 s) to DPH incorporated in liposomes. DPH:lipid weight ratio is 1:500. (D) The intensity changes at the 430-nm peak are plotted against time. A66W (●), F202W (▲) excited at 280 nm and A66W (○) excited at 360 nm.

with amphiphysin/arfaptin shape and examined its tubulation activity. The mutant ( $\Delta$ App), in which the entire appendage (Q59–Q88) was replaced with a helical stretch (AHLSSLQ) derived from arfaptin2 sequence (A152–Q159, Y155S), show the crystal structure of a canonical BAR-domain dimer as designed (Figure 5A and Supplementary Figure 7). The  $\Delta$ App could bind to liposomes (Figure 3) and cause tubulation to a lesser extent than the wild type and amphiphysin-BAR (Figure 5D and Supplementary Figure 4). As the diameter of the tubules reflects the membrane curvature if the section of the tube is circle, we measured the diameter of the tube to compare the curvature of the EndA1-BAR and its mutant-induced tubes. Despite the higher curvature of the concave surface, the  $\Delta$ App dimer induced larger diameter tubules than the wild type did, indicating a positive contribution of the wild-type hydrophobic ridge to drive membrane curvature. Taken all together, the hydrophobic ridge penetrates into the interfacial leaflet of the lipid bilayer

when the concave surface is in contact with the membrane and promotes membrane curvature formation.

#### The BAR domain is rigid enough to impose its intrinsic curvature on membrane

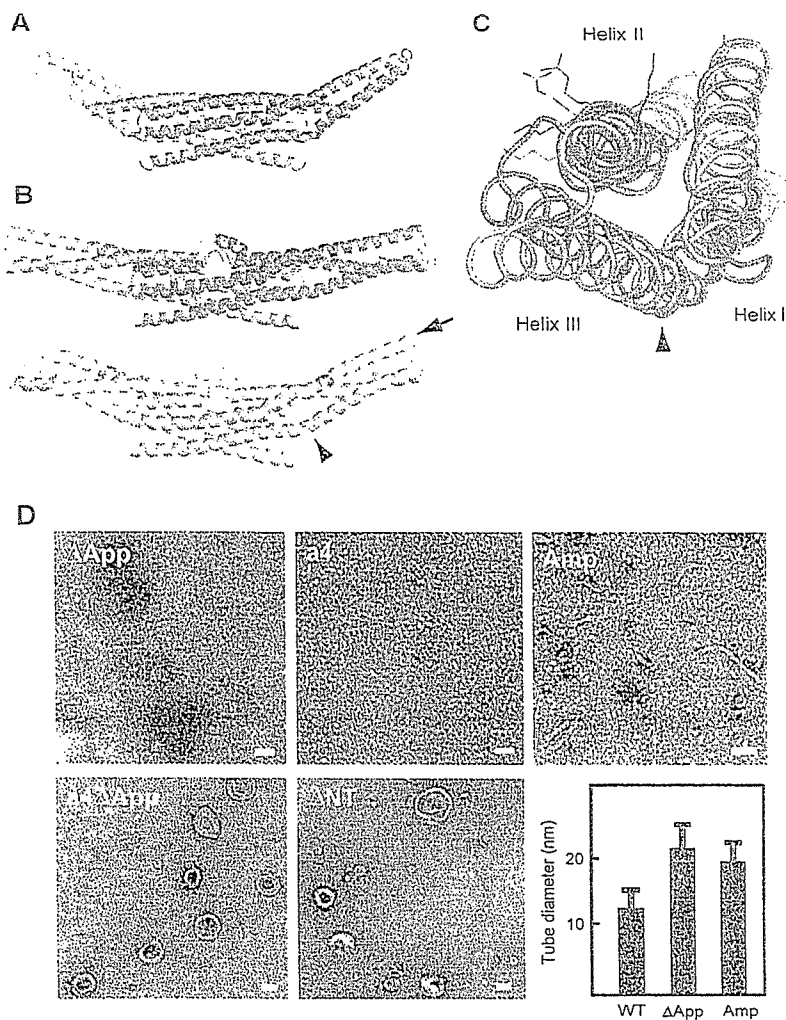
A simple model for the concave surface-driven mechanism is that each BAR domain dimer acts as a molecular mold that impresses its curved surface on the membrane. This model suggests that the membrane curvature approximately mirrors the curvature of the concave surface. Indeed, the diameters of tubules induced by amphiphysin,  $\Delta$ App (Figure 5D), SS and SSQ mutants (Figure 2) are compatible with the model-based prediction (see Supplementary Table II for statistical analysis). However, this model has an assumption that the dimer should be rigid enough to overcome the bending resistance of the membrane (Nossal and Zimmerberg, 2002; Farsad and De Camilli, 2003). To examine whether the molecular mold mechanism is feasible, we developed a straight BAR domain by inserting one helical pitch into the helix II in the proximal portion of the extending arm (QSAL is inserted between I154 and Q155). This mutation (a4) would compensate the unequal lengths between helix II and III in the arm, a common feature of the known BAR domain structures, and let the curved arm into a straight one. Although the a4 mutant was designed simply to straighten the curvature of the domain, the structure solved at 2.4 Å resolution shows that it actually has the very interesting property of a flexible arm rather than a rigid one (Figure 5B). Four monomers in the asymmetrical unit show deviation in the bending angles of arms. The blue and the green monomers have straight arms while the orange monomer shows a bending pattern similar to the wild type and the yellow monomer is an intermediate. The structural deviation almost exclusively occurs in the helix kink regions (Supplementary Figure 8), indicating that the arm can swing at least from the bend-free straight position to nearly the wild-type position.

The a4 mutant allowed us to examine how flexibility of the crescent-shaped main body of the BAR dimer affects the membrane curvature formation. The insertion of one helical pitch slightly distorts relative position of the helix II and III (Figure 5C), but does not largely rearrange the spatial positions of the residues on the concave surface of the arm (Supplementary Figure 8). Indeed, the a4 mutant and its appendage-lacking derivative (a4 $\Delta$ App) retained normal liposome binding activity (Figure 3). The a4 mutant vesiculated liposomes without any tubulation, while a4 $\Delta$ App lost these membrane-deforming activities (Figure 5D and Supplementary Figure 4). The concave surface-induced membrane deforming activity appeared to be lost in the a4 mutant, while the appendage's membrane insertion remained active. These results suggested that the rigidity of the crescent dimer structure is essential for liposome tubulation but not for vesiculation, although appendage insertion induces the vesiculation.

#### Roles for the amphipathic helix 0 of the N-BAR domain

The structure of a short amphipathic helix (helix 0) characterizing the N-BAR (Peter *et al*, 2004) can be resolved in the a4 mutant structure due to its tight crystal packing (Figures 5B and 6). The helix 0 is disordered in the wild type (Figure 6) and the  $\Delta$ App structures. The helix 0 has been





**Figure 5** Distinct liposome tubulation induced by endophilin-A1 BAR domain mutants. (A) Ribbon representation of a mutated EndA1-BAR dimer lacking the entire appendages ( $\Delta$ App, PDB ID: 1X04). The entire appendage (Q59–Q88) was replaced with a helical stretch (AHLSSLLQ) derived from arfapin2 sequence (A152–Q159, Y155S). Red, mutated segment. (B) Ribbon representation of the a4 mutant with swinging arms (PDB ID: 2D4C). One helical pitch was inserted into the helix II in the proximal portion of the extending arm (QSAL was inserted between I154 and Q155). Two dimers in the asymmetrical unit are shown separately. Red, inserted segment; magenta, helix 0. The bending patterns of the helix II and III varies among four monomers. An obvious kink in the helix III remains in the orange monomer (arrowhead, also in (C)). The residual curvature in the blue–green dimer is provided by the intersection of the monomers. (C) Superimposition of the a4 mutant monomer (orange one in (B)) and the wild-type monomer (blue) in the core region. A view from the distal end along the helix II (arrow in (B)) shows the maximum structural difference in these arms. Side chains of K171, 173 and R174 are shown. The helix III rotates  $12^\circ$  counterclockwise and shift 6 Å relative to the helix II at the distal end of the arm. The helix 0 and the core region are omitted. (D) Negatively stained liposome tubules induced by the BAR domains of endophilin mutants and amphiphysin.  $\Delta$ App, 7  $\mu$ M, incubated for 10 min; a4, 7  $\mu$ M, 10 min; a4 $\Delta$ App, 28  $\mu$ M, 10 min;  $\Delta$ NT, 21  $\mu$ M, 10 min; Amp, 7  $\mu$ M, 10 min. Note that a4, a4 $\Delta$ App, and  $\Delta$ NT do not induce liposome tubulation. Scale, 100 nm. The bar graph shows tubule diameter (mean and s.d.).

suggested to be helical only when the amphiphysin BAR domain binds to liposomes (Peter *et al*, 2004). The helix 0 displays the hydrophobic branch of T14, V17 and V21 on one side, while K12, K16 and E19 on the other side (Figure 6). The helix 0 is connecting with the Helix I by a flexible linker G23–G24–A25. Consistent with the previous report (Farsad *et al*, 2001), truncation of the helix 0 ( $\Delta$ NT) resulted in loss of liposome binding activity (Figure 3) and consequently abolished the tubulation (Figure 5D). In contrast, all the helix 0-containing mutants, including the A66D and the a4 $\Delta$ App showed intact liposome binding activity irrespective of their tubulation or vesiculation activities. These results indicate that the helix 0 in the endA1-BAR is critical for liposome binding and that the membrane binding of endA1-BAR via helix 0 is not sufficient to induce tubulation or vesiculation.

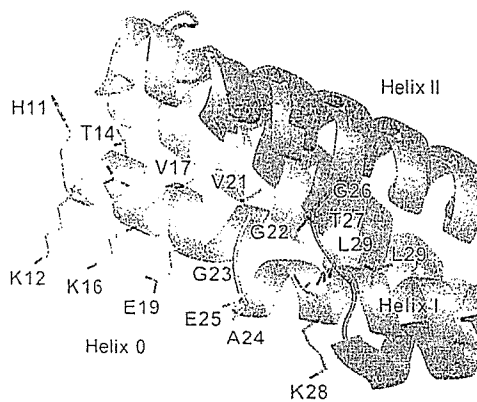
#### **BAR domain induces tubular membrane deformation *in vivo***

To explore the significance of the helix 0, the rigid crescent mold, and the appendage of endophilin-A1 BAR domain *in vivo*, we further examined the membrane deformation activity of endophilin-A1 BAR domain in cells (Figure 7). Human umbilical vascular endothelial cells (HUVECs) expressing endophilin-A1 lacking SH3 domain (residues 1–296, hereafter, EndA1-BAR296), which was C-terminally tagged with enhanced green fluorescence protein (EGFP), exhibited intracellular fibrous structure similar to those induced by other BAR domain-containing molecules (Kamioka *et al*, 2004; Itoh *et al*, 2005). Notably, these structures developed from the periphery toward the center of the cells dynamically and disappeared reversibly in living cells (Figure 7E and

Supplementary Movie 1). Furthermore, these GFP-marked structures were co-localized with *in vivo* biotin-labeled membrane (Figure 7D), indicating that EndA1-BAR296-induced fibrous structure seems to be a membrane invagination originated from the plasma membrane. These structures were found in other cells we tested (Figure 7C). In clear contrast,  $\Delta$ App,  $\Delta$ NT and a4 were incapable of inducing membrane deformation in cells, indicating the importance of helix 0, the rigid crescent shape, and the appendage of BAR domain for membrane deformation *in vivo*.

## Discussion

The endophilin-A1 BAR domain dimer consists of three sub-modules: the crescent-shaped main body, the helix 0 and the unique appendage. We tried to understand the functional roles for these sub-modules in the membrane curvature formation. In this study by determining the structure of

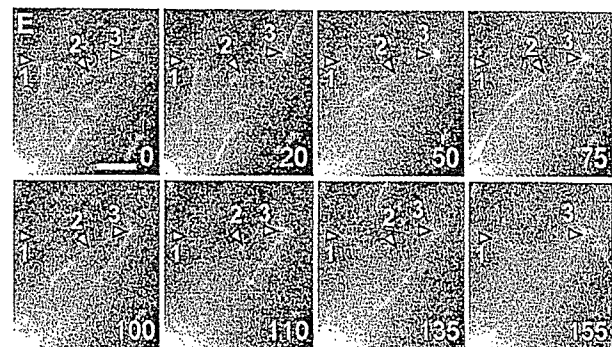
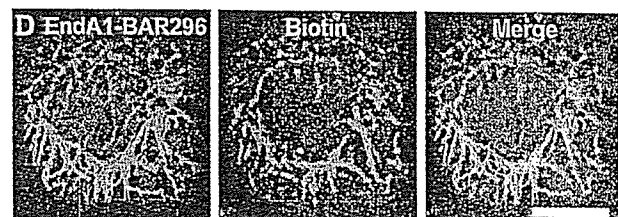
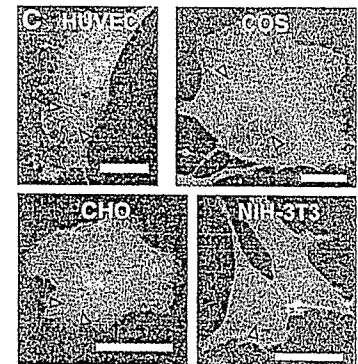
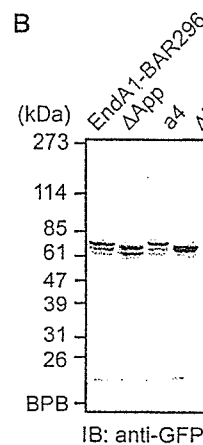
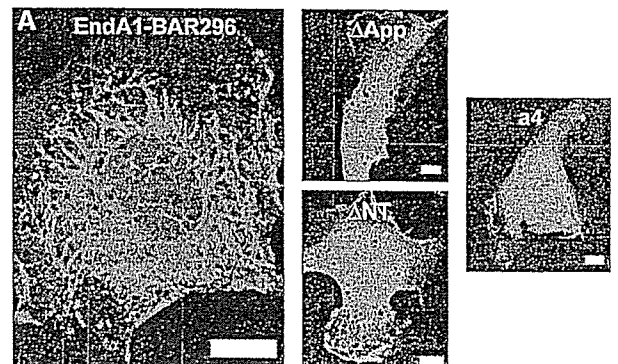


**Figure 6** Close-up of helix 0 in an a4 mutant monomer (orange). The same superimposition as in Figure 5C but viewed from the side and displays the helix 0. The helix 0 is disordered in the wild-type structure (blue). The side chains of N-terminal residues are shown (H11KATQKVSEKVGGAEGTKL29 in the a4 and G26TKL in the wild type). The amphipathic helix 0 is stabilized by hydrophobic interactions with the helix II and III and also by hydrogen bonds with a symmetrical molecule.

**Figure 7** Endophilin A1 BAR domain induces membrane tubulation *in vivo*. (A) HUVECs were transfected with plasmids expressing C-terminally EGFP-tagged EndA1-BAR296 (amino acid 1–296 of endophilin-A1),  $\Delta$ App, a4, and  $\Delta$ NT. Cells were GFP-imaged on an epifluorescence microscope (Olympus IX-71). Fibrous structures were observed exclusively in EndA1-BAR296-expressing cells. Scale, 10  $\mu$ m. (B) Protein expression of the EndA1-BAR296 and the mutants tagged with EGFP in transfected 293T cells were examined by immunoblotted with anti-GFP antibody. (C) Cells indicated were similarly transfected to (A). Arrowheads indicate the fibrous structures. Scale, 20  $\mu$ m. (D) Live HUVECs expressing EGFP-tagged EndA1-BAR296 were biotinylated with sulfo-NHS-biotin for 10 min and chased for further 10 min. Covalently bound biotin was visualized using Alexa633-streptavidine. Fluorescence images for EGFP (left), Alexa633 (center), and merge (right) are shown. Scale, 10  $\mu$ m. (E) A time lapse images of HUVECs expressing EGFP-tagged EndA1-BAR296 were obtained at the time point (seconds) after the observation (Supplementary Movie 1). EGFP-marked structure grows from the cell periphery towards the center of the cell. Notably, both extension and retraction of GFP-marked structure is observed (numbered arrow heads indicate each extending/retracting structure). Scale, 5  $\mu$ m.

endophilin-A1 BAR domain and developing mutants that were critical for the sub-module structure, we have explored the roles of sub-modules.

Here, we show that the structural rigidity of the crescent-shaped main body is critical for membrane tubulation. The BAR dimer is sufficiently rigid to overcome the bending resistance of the membrane and to be scaffolds for the tubulation (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). The insertion of one helical-pitch into the helix II at distal to the kink brings flexibility to the dimer (a4 mutant). The relative position of the three helices in the



mutant arm was not changed in a4 mutant irrespective of the bend levels (Supplementary Figure 8). The mutant arm behaves as a rigid body and its structure changes only in the vicinity of the helix kinks when it swings. Therefore, it is unlikely that the flexibility of the mutant dimer can be a result of weakened inter-helix interactions in the arm. Moreover, we could not find any specific structural features in the kink region that might explain the flexible hinge in the swinging-arm mutant as well as the rigid bend in the wild-type BAR dimers of endophilin, amphiphysin, and arfaptin.

In this study, for the first time we could determine the structure of the N-terminal amphipathic helix (helix 0) using a swinging-arm mutant. Our mutant and previous mutation analyses indicated that the N-terminal helical sequence of endophilin-A1 is indispensable for liposome binding (Farsad *et al*, 2001), whereas that of amphiphysin is important but not essential for liposome binding and tubulation (Peter *et al*, 2004). The BAR domain of endophilin-A1 is an acidic polypeptide and the cluster of positive charge at the distal end of the arm is not prominent (Figure 1A). This property can explain the critical role for the helix 0 of the EndA1-BAR in liposome binding by providing additional basic residues. The helix 0 structure suggests that K12, K16 and possibly K8 are in a suitable position for cooperation with the positive charge cluster at the distal end. The amphipathic nature of the helix 0 implies that it can also insert into the membrane and facilitate the membrane curvature formation (Peter *et al*, 2004; Gallop and McMahon, 2005; McMahon and Gallop, 2005). Loss of the membrane-deforming activities of the A66D mutant (Figure 2) and the a4ΔApp mutant (Figure 5D) accounts for the additional mechanism for membrane deformation in addition to the membrane insertion of the helix 0.

The N-BAR of endophilins has one additional step to tubulate membrane. Here, we show that the hydrophobic ridge of the endophilin-specific appendage is inserted into the contacting membrane surface. Our data suggested that the entire ridge of the wild-type BAR domain, about 8 Å in height, is embedded in the layer of lipid head-groups of the contacting membrane leaflet. The embedding of the ridge into the membrane is consistent with the local spontaneous curvature mechanism that is reported very recently (Zimmerberg and Kozlov, 2006). As a protruding structure found in epsin1 induces liposome tubulation by being inserted to one leaflet of the lipid bilayer (Ford *et al*, 2002), the penetration of the hydrophobic ridge can drive the positive curvature by causing asymmetrical expansion of the surface area between two leaflets as shown in Figure 8 (Farsad and De Camilli, 2003).

We further explored the importance of the ridge, rigid crescent shape, and helix 0 in cells. We for the first time showed that N-BAR domain induced membrane invaginations originated from plasma membrane, although other BAR-containing molecules have been reported to induce similar invaginations (Itoh *et al*, 2005). Neither mutant that lacked either the ridge or the helix 0 nor flexible mutant formed the tubular invaginations in cells, indicating the significance of these sub-module structure in cells as suggest by *in vitro* studies. We constructed a series of endophilin-A1-EGFP expression plasmids to delineate the domain for the membrane invagination. Full-length endophilin-expressing cells did not show any tubular formation. Because endophilin consists of BAR domain and an SH domain, SH3-binding molecule such

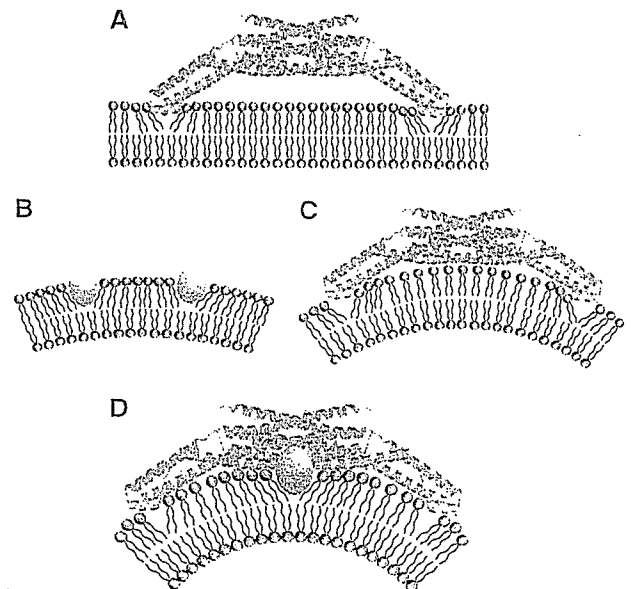


Figure 8 Two potential mechanisms for driving membrane curvature by endophilin-A1. (A) Kissing adhesion of an N-BAR domain on planar lipid bilayer. The helix 0 is essential for the membrane binding. Membrane insertion of the helix 0 is supposed. (B) Insertion of hydrophobic portions of macromolecules into one leaflet can create bilayer surface discrepancy that causes membrane curvature. (C) The simple N-BAR domain, such as amphiphysin and ΔApp, induces membrane curvature by impressing the concave surface onto the membrane. The rigidity of the molecule is required for this mechanism. (D) To drive membrane curvature, the endophilin N-BAR domain uses both the rigid crescent shape-mediated deformation and the insertion of hydrophobic ridge on the concave surface in addition to kissing adhesion of N-BAR to membrane surface.

as dynamin may inhibit the extension of membrane invagination. This possibility has been suggested in the membrane invagination found in FBP17 and amphiphysin (Kamioka *et al*, 2004; Itoh *et al*, 2005).

Collectively, EndA1-BAR uses two newly identified mechanisms to drive positive membrane curvature in addition to the essential binding capacity of helix 0 to the membrane: one by the scaffold mechanism common to the BAR domains and the other by the local spontaneous curvature mechanism caused by the membrane insertion of the ridge (Figure 8D). The ridge, which occupies the bottom of the concave lipid-binding surface, may not work until the main body of the BAR dimer localizes itself to a curved membrane. The ridge then inserts into the bilayer roughly perpendicular to the main body, and thus both deformations will occur in the same direction.

## Materials and methods

### Protein expression and purification by CRECLE

cDNAs encoding BAR domains (amphiphysin1, 1–239; endophilin-A1, 1–247; endophilin-B1, 1–246 in amino-acid residues) were amplified by PCR from a human brain cDNA library. Recombinant proteins were expressed in *Escherichia coli* as GST-fusions using the pGEX6p3 vector, purified by glutathione-Sepharose, cleaved from the GST-tag using Prescission protease (Amersham Biosciences), and further purified by ion-exchange chromatography (Yamagishi *et al*, 2004). The final polypeptide contained an artificial linker

sequence of GPLGS at the N-terminus. EndA1-BAR proteins except for F202W and a4 mutants were purified by crystallization during Prescission protease cleavage. The method, crystallization by regulated cleavage of large hydrophilic tag (CRECLE), was as follows. Purified GST fusions were concentrated to 20–30 mg/ml in an elution buffer (20 mM glutathione, 100 mM Tris-HCl, pH 8.0, 10 mM DTT, 1 mM EDTA, 1 mM EGTA) and then cleaved by a low concentration of prescission protease (1 U/mg protein or less) at 4°C. Slow increase in the tag-free protein concentration might be suitable for crystallization and more than a half of EndA1-BAR protein could be recovered as 20–100 µm microcrystals. They were washed with a low-salt buffer (20 mM HEPES, pH 7.4, 2 mM DTT, 0.2 mM EDTA, 0.2 mM EDTA) and resolved into a high-salt buffer (350 mM NaCl in the low-salt buffer) and used for further analyses.

#### Protein crystallization

Seleno-methionine (S-Met) derivatives of the EndA1-BAR domain and its appendage-less mutant ( $\Delta$ App) were produced in B834(DE3)pLysS cells using Overnight Express Autoinduction System 2 (Novagen). To make X-ray grade crystals in a cryo-ready condition, modified high salt buffer (50 mM HEPES, pH 7.4, 300 mM NaCl, 100 mM KI, 28% ethylene glycol, 5% glycerol, 25 mM DTT) was used. Crystals of 1 mm size were formed by dialysis against 50 mM CHES, pH 9.5, 260 mM NaCl, 28% ethylene glycol, 5% glycerol, 25 mM DTT, 0.4% benzamidine·HCl at 4°C and were flash frozen at 100 K. Crystals could also be grown by vapor diffusion from a similar protein solution using distilled water as the bath solution. The crystals were equilibrated in 50 mM HEPES, pH 7.4, 150 mM NaCl, 25 mM DTT, 0.4% benzamidine·HCl, 5% PEG 8000 and the saturated amount of xylitol as a cryoprotectant. Some of the crystals were soaked with 0.5 mM oleoyl-L- $\alpha$ -lysophosphatidic acid (Sigma) or malonyl-CoA (Sigma) for 4 days with daily change for the substrates. The a4 mutant crystals were grown by sitting-drop vapour diffusion using a bath solution containing 100 mM HEPES, pH 7.2, 200 mM calcium acetate, 10 mM DTT and 20% (w/v) PEG3350 at 20°C and then flash frozen after brief immersion in the same solution containing 16% DMSO. The wild type and the  $\Delta$ App mutant crystals belong to the same space group  $I4_1$  and contain one monomer molecule in the asymmetric unit (Supplemental Figure 1). The a4 crystal belongs to  $P2_1$  and contains two dimers in the asymmetric unit.

#### Structural determination

The EndA1-BAR structure was determined using the multiple anomalous dispersion (MAD) method. Multiple-wavelength X-ray diffraction data sets were collected from a single Se-Met crystal (crystal I) at SPring-8 beamline BL44B2 (Supplementary Table 1). Single wavelength data sets of another crystal (crystal II) and of a  $\Delta$ App crystal used for the refinement were collected at BL45PX. The data set for the a4 mutant was collected at BL38B1. All diffraction data sets were collected at 90 K and were processed using HKL2000 suite (Otwinowski and Minor, 1997). The seven positions out of 10 expected selenium atoms were identified by SOLVE (Terwilliger and Berendzen, 1999). The initial phases calculated by SOLVE with a figure of merit of 0.59 at 3.2 Å resolution were further improved by RESOLVE (Terwilliger, 1999). The density modified MAD map (Supplementary Figure 1) had sufficient quality to trace the polypeptide chain except for the N-terminus and the loop region of the appendage. The model was built with TURBO-FRODO (Roussel and Cambillau, 1996) and refined to the resolutions of 3.1 Å by CNS (Brunger *et al*, 1998). The final model includes 210 residues (residues 26–71 and 84–247), and has an  $R$  factor of 23.6% ( $R_{\text{free}}$  of 26.4%). The  $\Delta$ App structure was solved by molecular replacement by MOLREP in the CCP4 suite (CCP4, 1994) and refined to the resolution of 2.9 Å by CNS. The simulated annealing omit electron density map calculated by CNS confirmed the continuous  $\alpha$ -helical structure of the replaced region as designed (Supplementary Figure 7). The final model includes 200 amino-acid residues and has an  $R$  factor of 23.8% ( $R_{\text{free}}$  of 26.9%). The a4 mutant structure was solved by molecular replacement using the central core of the EndA1-BAR as a starting model and the arms were manually built (Supplementary Figure 7). The structure was refined to the resolution of 2.4 Å by CNS with an  $R$  factor of 21.5% ( $R_{\text{free}}$  of 26.9%). Main-chain dihedral angles of all non-glycine residues of these three models lie in allowed regions of the Ramachandran plot, with 94.3% for the EndA1-BAR, 94.1% for the

$\Delta$ App mutant, and 96.4% for the a4 mutant in most-favored regions, respectively. Graphical representations were prepared using the programs TURBO-FRODO, MOLSCRIPT (Kraulis, 1991), RASTER3D (Merritt and Bacon, 1997), GRASP (Nicholls *et al*, 1991) and Pymol (DeLano, 2002).

#### Liposome binding and tubulation assays

Liposome sedimentation assay and tubulation assay were as earlier described (Peter *et al*, 2004 see also McMahon lab protocols: [http://www2.mrc-lmb.cam.ac.uk/NB/McMahon\\_H/group/techniqs/techniqs.htm](http://www2.mrc-lmb.cam.ac.uk/NB/McMahon_H/group/techniqs/techniqs.htm)) with slight modifications. Briefly, Folch fraction 1 (Sigma) was used as the lipid source and liposome suspension, 1 mg/ml in liposome buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT) was made by sonication. Freshly purified BAR domain proteins were diluted at about 1 mg/ml in the liposome buffer and ultracentrifuged at 400 000 g for 10 min just before use. No crystallization occurred at this or lower concentrations. For sedimentation assays, 20 µg proteins were mixed with 25 or 75 µg liposomes in 100 µl of the liposome buffer, incubated for 10 min on ice and ultracentrifuged at 200 000 g for 10 min. For tubulation assays, 400 µg/ml proteins were mixed with an equal volume of 400 µg/ml liposomes, left for 10 s to 30 min at room temperature, and then processed for negative staining. Judging from the liposome sedimentation and the tryptophan fluorescence assays, this protein to lipid ratio ensured nearly saturated protein-liposome binding. Magnification was calibrated using a grating replica of 2160/mm.

#### Tryptophan fluorescence and FRET assay

Fluorescence emission spectra were recorded with a Hitachi F-4500 fluorescence spectrophotometer (Ohki *et al*, 2004). For tryptophan fluorescence assays, 140 µg/ml tryptophan-containing mutants were mixed with 0–200 µg/ml liposomes in the liposome buffer, incubated for 3 min, and excited at 280 nm. For FRET assays, DPH-liposomes were made by adding DPH (Molecular Probe) into lipid solution (1:500 to lipid, w:w). The fluorescence of DPH-liposomes (200 µg/ml) excited at 280 nm was scanned from 400 to 500 nm at 1-min intervals. The first measurement of the 430-nm DPH peak was obtained at about 30 s after mixing with mutant proteins (100 µg/ml).

#### Cell culture, transfection and surface biotinylation

HUVECs were purchased from Kurabo and cultured in HuMedia-EG2 as described previously (Sakurai *et al*, 2006). 293T cells, CHO cells, Cos7 cells, and NIH-3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum as described previously (Kamioka *et al*, 2004). Cells were transfected using LipofectAMINE 2000 (Invitrogen). Live HUVECs were biotinylated with 5 mM sulfo-NHS-biotin (Pierce) in Opti-MEM (Invitrogen) for 10 min. They were washed once with Opti-MEM and chased for 10 min with the normal culture medium, and fixed with 2% formaldehyde after a brief wash with Opti-MEM containing 1/20 volume of Avidin D blocking solution (Vector Laboratory) to reduce the cell surface background staining. HUVECs were permeabilized with cold MeOH and biotin was visualized using Alexa633-streptavidine (Molecular Probe).

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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#### Competing interests statement

The authors declare that they have no competing commercial interests in relation to this work.

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