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Cardiovascular Drug Reviews
Vol. 25, No. 4, pp. 333–341

Serofendic Acid, a Substance Extracted from Fetal Calf Serum, as a Novel Drug for Cardioprotection

Masaharu Akao,[1] Toshihiro Takeda,[1] Toru Kita,[1] Toshiaki Kume,[2] and Akinori Akaike[2]

[1] *Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan;* [2] *Department of Pharmaceutical Science, Kyoto University Graduate School of Pharmacology, Kyoto, Japan*

Keywords: Calf serum — Cardioprotection — Ischemia — Mitochondria — Reperfusion — Serofendic acid.

ABSTRACT

We previously identified a novel endogenous substance, serofendic acid, from a lipophilic extract of fetal calf serum. The compound, a low-molecular-weight sulfur-containing atisane-type diterpenoid, exhibited potent protective action against neurotoxicity induced by glutamate, nitric oxide, and oxidant stress. We investigated whether this substance has a cardioprotective effect. Primary cultures of neonatal rat cardiac myocytes were exposed to oxidant stress (H_2O_2) to induce cell death. Pretreatment with serofendic acid significantly suppressed cell death induced by H_2O_2, and the cytoprotective effect was closely associated with the preservation of mitochondrial function. Serofendic acid inhibited H_2O_2-induced loss of mitochondrial membrane potential in a concentration-dependent manner (with saturation by 100 μM), by attenuating matrix calcium overload and intracellular accumulation of reactive oxygen species. The protective effect of serofendic acid was comparable to that of a mitochondrial ATP-sensitive potassium (mitoK$_{ATP}$) channel opener, diazoxide. Furthermore, mitoK$_{ATP}$ channel blocker, 5-hydroxydecanoate, abolished the protective effect of serofendic acid. Serofendic acid and diazoxide, administered together, at 100 μM each, had no additive effects. Thus, serofendic acid inhibited the oxidant-induced mitochondrial death pathway, presumably through activation of the mitoK$_{ATP}$ channel. In conclusion,

Address correspondence and reprint requests to: Masaharu Akao, MD, PhD, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81-75-751-3194; Fax: +81-75-751-4284; E-mail: akao@kuhp.kyoto-u.ac.jp
Conflict of Interest: The authors have no conflict of interest.

serofendic acid appears to protect cardiac myocytes from oxidant-induced cell death by preserving the functional integrity of mitochondria. Our findings suggest that serofendic acid may represent a novel candidate for cardioprotective therapy in ischemia/reperfusion injury.

INTRODUCTION

Ischemic heart disease, as the underlying cause of acute myocardial infarction (AMI), congestive heart failure, arrhythmias, and sudden cardiac death, is the leading cause of morbidity and mortality in all industrialized nations. As the population grows older and comorbidities such as obesity and diabetes become more prevalent, the enormous public health burden caused by ischemic heart disease is likely to increase even further.

The importance of limiting myocardial ischemia/reperfusion injury has been appreciated for more than 3 decades. In 1971, Braunwald et al. published a landmark study (Maroko et al. 1971) in which they proposed the groundbreaking idea that the extent and severity of tissue damage after coronary occlusion were not predetermined at the onset of ischemia but could be modified by therapeutic manipulations applied during ischemia (Maroko et al. 1971). This concept produced a major paradigm shift, and enormous effort to identify cardioprotective therapies has been made during the last 3 decades. Especially, the infarct size-limiting effect of ischemic preconditioning, a mechanism by which brief episodes of ischemia produce protection against subsequent longer ischemic insults (Murry et al. 1986), was remarkable. Up to now, a number of experimental interventions (both pharmacologic and nonpharmacologic) have been claimed to limit myocardial infarct size in experimental animals. Unfortunately, few of these results have been reproducible and none has been translated into clinical practice.

Mitochondria play critical roles in cell death in response to a variety of stresses such as myocardial ischemia/reperfusion (Green and Kroemer 2004; Kroemer et al. 1998; Weiss et al. 2003). Opening of the mitochondrial permeability transition pore (MPTP), a nonspecific pore, that opens at the contact site between outer and inner mitochondrial membranes, results in the loss of mitochondrial inner membrane potential ($\Delta\Psi_m$), matrix swelling, and the release of cytochrome *c* and other proapoptotic factors that lead to cell death (Crompton 1999; Crow et al. 2004; Halestrap et al. 2004). Mitochondrial matrix calcium ($[Ca^{2+}]_m$) overload and reactive oxygen species (ROS) favor MPTP opening (Brookes et al. 2004). Inhibition of MPTP opening by preventing $[Ca^{2+}]_m$ overload and ROS generation has been an effective strategy for the protection of hearts from ischemia/reperfusion injury (Bouchier-Hayes et al. 2005)..

We have shown that ATP-sensitive potassium channels located in the inner mitochondrial membrane (mitoK$_{ATP}$ channels) play a central role in the signaling cascade of protection against oxidative stress in the cardiac ventricular myocytes (Akao et al. 2001, 2002) and the cerebellar granule neurons (Teshima et al. 2003a, 2003c). MitoK$_{ATP}$ channels prevent $[Ca^{2+}]_m$ overload and ROS generation, thereby inhibiting the MPTP opening in both types of cells (Akao et al. 2003a, 2003b; Murata et al. 2001; Teshima et al. 2003c). Diazoxide, a selective opener of mitoK$_{ATP}$ channels, has been shown to have protective effects against myocardial ischemia/reperfusion both *in vitro* (Garlid et al. 1997; Liu et al. 1998) and *in vivo* (Fryer et al. 2000; Miura et al. 2000). Unfortunately, the clinical use of this agent

has been hampered due to unwanted side effects, such as excessive hypotension, edema, and so on. On the basis of these discoveries, new cardioprotective drugs should have been discovered and made clinically available a long time ago.

In search for novel protective substances of mammalian origin, we purified and isolated a novel factor from an extract of fetal calf serum based on its ability to protect cultured cortical neurons from nitric oxide neurotoxicity (Kume et al. 2002). We found that this substance has a cardioprotective effect.

CHEMISTRY

As stated above, we purified a novel neuroprotective substance from fetal calf serum (Kume et al. 2002). Briefly, we searched for the active compounds in the ether extract of fetal calf serum (EE-FCS) by testing the effects of HPLC fractions from EE-FCS in an *in vitro* bioassay with cultured cortical neurons. Lipophilic substances in heat-inactivated fetal calf serum were extracted with diethyl ether, and the residue of EE-FCS was subjected to reversed phase HPLC. Serial fractionation steps resulted in the presence of two compounds with the same molecular weight 382 in the active fraction. We assumed that these compounds were stereoisomers with the same planar structure.

To determine the chemical structure of the isolated compounds, we performed a large-scale extraction from 250 L of fetal calf serum. Two peaks of prominent ions in the purified fraction with final yields of 1.7 and 1.4 mg were confirmed.

The ^{1}H nuclear magnetic resonance (NMR) spectra revealed the alicyclic nature of the compounds and demonstrated the presence of a methylsulfoxide group. A standard set of two-dimensional NMR experiments revealed that the two compounds possessed same planar structure. Chemical structure established by these experiments was 15-hydroxy-17-methylsulfinylatisan-19-oic acid, a sulfur-containing atisane-type diterpenoid (Fig. 1). The identified compounds were named "serofendic acid" (SFA), since they were isolated from serum (sero-), showed cytoprotective effect (-fend), and possessed carboxylic acid. The discovery of atisane derivatives has not been documented in animals, although a few atisane derivatives of plant origin, distinct from SFA, have been reported (Appendino et al. 2000).

Our unpublished data show that SFA is contained in fetal calf serum in a considerable amount, but the content in adult bovine is below detectable level. Similarly, we were unable to detect SFA in adult human serum, although there is a possibility that fetal human

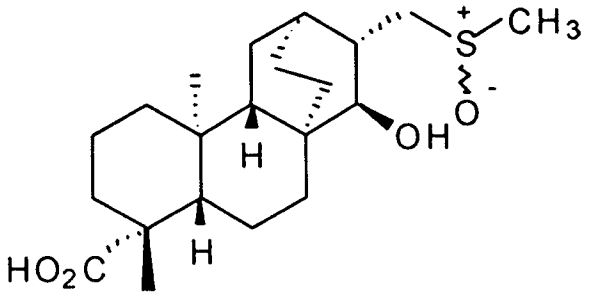

FIG. 1. Chemical structure of serofendic acid.

serum may contain SFA. The biosynthesis or the metabolism of SFA have not yet been investigated.

PHARMACOLOGY

Neuroprotective Action

The compound exhibited the ability to protect cultured neurons from a variety of cellular stress, such as induced by glutamate, nitric oxide, or H_2O_2 (Akaike et al. 2003; Kume et al. 2002; Kume et al. 2005, 2006; Osakada et al. 2004; Taguchi et al. 2003). The neuroprotective effect of SFA has been demonstrated also in a rat model of Parkinson disease, where oxidative stress is involved (Inden et al. 2005). Furthermore, we discovered recently that SFA, by intracerebroventricular administration, reduces infarct size and ameliorated the neurologic deficit score after cerebral infarction in a rat model of middle cerebral artery occlusion (unpublished). Suppression of intracellular ROS generation may constitute an important mechanism of the neuroprotective actions of SFA, since the compound exhibits hydroxyl radical-scavenging activity in electron spin resonance analysis (Kume et al. 2002). The suggested mechanisms of neuroprotective action of SFA were summarized in our previous publications (Akaike et al. 2003; Kume et al. 2004).

Cardioprotective Activity

Given that oxidative stress is also responsible for the tissue injury during myocardial ischemia/reperfusion, we hypothesized that SFA may have cardioprotective effects against ischemia/reperfusion injury. In isolated neonatal rat cardiac myocytes, SFA suppressed the cell death induced by H_2O_2, as evidenced by the fewer TUNEL-positive nuclei, preservation of nuclear morphology, and better MTS cellular viability assay results in the SFA-treated preparations (Takeda et al. 2006).

To examine whether preservation of $\Delta\Psi_m$ is associated with the cardioprotective effects of SFA, we assessed the H_2O_2-induced changes of TMRE, a fluorescent marker of $\Delta\Psi_m$, using fluorescence-activated cell sorter (FACS) analysis. The majority of cells in the control group (Fig. 2A, panel C) belonged to a population with a high TMRE fluorescence level (indicated by vertical dashed line). Exposure to H_2O_2 shifted the predominant population to a lower TMRE fluorescence (Fig. 2A, panel H). SFA protected against the H_2O_2-induced loss of $\Delta\Psi_m$, preserving a population of cells with a normal $\Delta\Psi_m$ level (Fig. 2A, panel SFA). These observations were quantitated by plotting the percentage of cells with high TMRE (>300, in this case), as shown in Figure 2B. Exposure to 100 μM H_2O_2 for 1 h resulted in mitochondrial depolarization, whereas SFA prevented the loss of $\Delta\Psi_m$ in a concentration-dependent manner. The $\Delta\Psi_m$-preserving effect of SFA reached its maximum level at 100 μM. We further compared the protective effects of SFA with those of diazoxide, a mitoK_{ATP} channel opener. In isolated cardiac myocytes, we previously reported that diazoxide prevents the loss of $\Delta\Psi_m$ induced by oxidative stress in a concentration-dependent manner (Akao et al. 2001). As shown in Figure 2C, the protective effect of 100 μM SFA was comparable to that produced by 100 μM diazoxide [maximal protective concentration of diazoxide (Akao et al. 2001)] in preventing the loss of $\Delta\Psi_m$ induced by

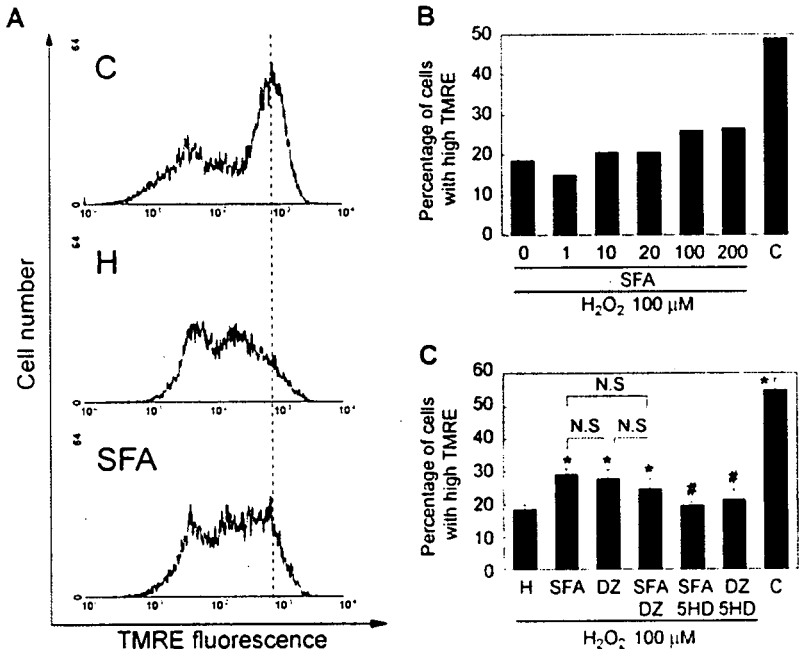

FIG. 2. Mitochondrial inner membrane potential ($\Delta\Psi_m$) in neonatal rat cardiac myocytes. (**A**) FL-2 histograms of FACS data from TMRE-loaded cells are shown. C, Control cells; H, cells exposed to 100 μM H_2O_2 for 1 h; SFA, cells pretreated with 100 μM SFA for 30 min followed by 100 μM H_2O_2 for 1 h. In all of the histograms, the position of the major population of control group is indicated by a vertical dashed line. (**B**) Representative data of the percentage of cells that maintain high (>300) TMRE fluorescence. Cells were pretreated with various concentrations of SFA for 30 min, followed by 100 μM H_2O_2 for 1 h. C, Control cells. SFA preserved $\Delta\Psi_m$ in a concentration-dependent manner. (**C**) Summarized data of the percentage of cells that maintain high (>300) TMRE fluorescence. Cells were pretreated with various drugs for 30 min followed by 100 μM H_2O_2 for 1 h. SFA, 100 μM SA; DZ, 100 μM diazoxide; 5HD, 500 μM 5-hydroxydecanoate. $^*P < 0.05$ versus H. $\#P < 0.05$ versus corresponding 5HD-absent group. N.S., not significant.

100 μM H_2O_2. The protection afforded by either SFA or diazoxide was completely blocked by a mitoK_{ATP} channel blocker, 5-hydroxydecanoate [5HD, 500 μM (Akao et al. 2001)]. Co-application of 100 μM diazoxide and 100 μM SFA had no additive effect (Fig. 2C), since the protective effect of the combined treatment did not exceed the effect of each single drug.

To further confirm the protective effect of SFA in preventing the loss of $\Delta\Psi_m$, we examined the time-dependent changes of $\Delta\Psi_m$ in single cells (Fig. 3). Time-lapse confocal analysis of cardiac myocytes loaded with TMRE was performed at 2-min intervals. Time-lapse scanning began immediately after the application of H_2O_2. At first, we confirmed that TMRE fluorescence did not change during the 60 min of observation in the control group (Fig. 3A, panels C). In contrast, cells treated with H_2O_2 progressively lost their red fluorescence intensity, indicating the irreversible loss of $\Delta\Psi_m$ (Fig. 3A, panels H). TMRE fluorescence was remarkably preserved in the SFA-treated group (Fig. 3A, panels SFA). Figure 3B shows the average of TMRE fluorescence intensity from 25 randomly selected cells in each group, indicating the significant protective effects of SFA.

Furthermore, we showed that the preservation of mitochondrial integrity was most likely achieved by the partial inhibition of $[Ca^{2+}]_m$ overload and ROS accumulation (Takeda et al. 2006).

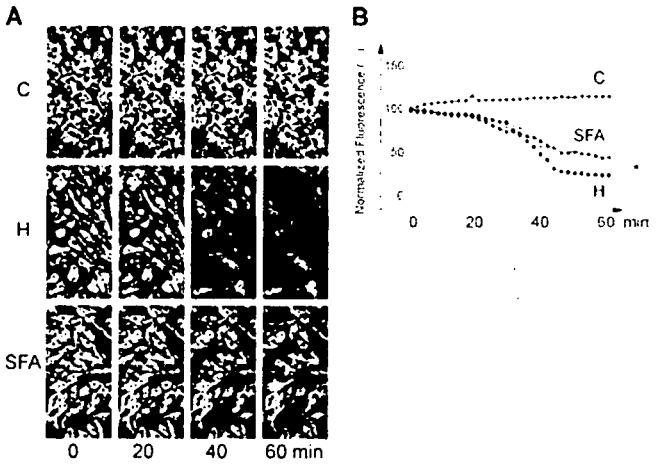

FIG. 3. Time-lapse analysis of $\Delta\Psi_m$ loss in neonatal rat cardiac myocytes. (**A**) Representative sequential images of TMRE fluorescence in each group. C, Control cells; H, cells exposed to 50 μM H_2O_2 for 1 h; SFA, cells pretreated with 100 μM SFA for 30 min followed by 50 μM H_2O_2 for 1 h. (**B**) Time course of TMRE fluorescence of 25 cells randomly selected in each group. (**C**) Mean fluorescence intensity from 25 cells randomly and prospectively selected in each group. $^*P < 0.05$ versus H at the end of the experimental period.

Suggested Mechanisms of Action

Prevention of MPTP by SFA

In recent studies, ROS generation and $[Ca^{2+}]_m$ overload have been proposed to explain the pathogenesis of ischemia/reperfusion injury of the heart (Griendling and Alexander 1997; Weiss et al. 2003). ROS and $[Ca^{2+}]_m$ are the most important inducers of MPTP opening. A growing body of evidence supports the concept that the inhibition of MPTP is an effective and promising strategy to prevent ischemia/reperfusion injury of the heart (Halestrap et al. 2004; Hausenloy et al. 2003; Murphy 2004; Weiss et al. 2003). We showed that SFA prevents MPTP opening, as reported by the preservation of the cell population with fully polarized (intact) $\Delta\Psi_m$ levels (Takeda et al. 2006). Notably, SFA only partly suppressed the increases of $[Ca^{2+}]_m$ and ROS, but this partial inhibition might decrease the number of cells that reach the threshold of the catastrophic loss of $\Delta\Psi_m$ (Takeda et al. 2006). In neurons, SFA similarly inhibited loss of $\Delta\Psi_m$ induced by glutamate exposure, but did not affect glutamate-induced increase in intracellular Ca^{2+} (Kume et al. 2006). In both types of cells, preservation of $\Delta\Psi_m$ level should be directly associated with the cytoprotective effect, but the underlying mechanisms may not necessarily be the same in neurons and cardiac myocytes.

As reported by us previously, oxidant stress produces a stereotyped progression of cellular changes in cardiac myocytes (Akao et al. 2003b). We call the first phase "priming": mitochondria undergo $[Ca^{2+}]_m$-dependent morphological changes, but $\Delta\Psi_m$ remains unchanged. The next phase is a sudden dissipation of $\Delta\Psi_m$ mediated by the opening of MPTP ("depolarization" phase); eventually, cells break up into smaller fragments ("fragmentation" phase). SFA markedly decreases the likelihood that cells would undergo priming: $[Ca^{2+}]_m$ overload is attenuated, and, consequently, many mitochondria remain fully polarized. SFA not only decreases the number of cells undergoing $\Delta\Psi_m$ depolarization, but also delays the

onset of $\Delta\Psi_m$ loss, whereas it does not change the duration of depolarization in unprotected cells (Takeda et al. 2006). This mode of action is equivalent to that of the mitoK$_{ATP}$ channel opener diazoxide (Akao et al. 2003a), raising the possibility that the cytoprotective effects of SFA are, directly or indirectly, mediated by the mitoK$_{ATP}$ channel.

SFA and mitoK$_{ATP}$ channel

Cardioprotective effect can be recruited by mitoK$_{ATP}$ channel openers, and mitoK$_{ATP}$ channel blockers (5HD or glibenclamide) prevent both preconditioning and pharmacological cardioprotection (Gross 1995; O'Rourke 2000). Furthermore, mitoK$_{ATP}$ channel opening prevents mitochondrial injury, presumably by inhibiting the opening of MPTP (Akao et al. 2003a, 2003b). MitoK$_{ATP}$ channel activation induces partial and modest $\Delta\Psi_m$ depolarization, thereby reducing the driving force for calcium uptake by mitochondria and preventing $[Ca^{2+}]_m$ elevation (Murata et al. 2001). This is further supported by the observation that partial $\Delta\Psi_m$ depolarization elicited by the overexpression of uncoupling protein-2 also protected cardiac myocytes (Teshima et al. 2003b). The protective effect of SFA was comparable to that of diazoxide, and there was no additive effect with co-application of SFA and diazoxide. Furthermore, the mitoK$_{ATP}$ channel blocker, 5HD, abolished the protective effect of SFA (Takeda et al. 2006). These results strongly suggest that the protective effect of SFA may be mediated by the activation of mitoK$_{ATP}$ channels.

SFA and mitochondrial apoptotic pathway

We recently demonstrated that SFA prevented the loss of $\Delta\Psi_m$ following glutamate exposure in cultured cortical neurons (Kume et al. 2006). SFA reduced the activation of caspase-3 induced by glutamate, and directly inhibited the activity of recombinant human caspase-3, -7, and -8. These results indicate that SFA prevents glutamate-induced mitochondrial apoptotic pathway in cultured neurons by preventing the loss of $\Delta\Psi_m$ and reduction of caspase-3 activation. In good agreement with these effects of SFA, the anti-apoptotic properties of mitoK$_{ATP}$ channel agonists have also been demonstrated both in cardiac myocytes (Akao et al. 2001, 2002; McCully et al. 2002) and neurons (Liu et al. 2002; Teshima et al. 2003a, 2003c).

FUTURE PROSPECTS

Many pharmacological agents and strategies have been administered for cardiac protection during acute myocardial infarction (Kloner and Rezkalla 2004). However, none has been translated into clinical practice (Bolli et al. 2004). Therapeutic interventions designed to prevent MPTP opening during ischemia/reperfusion hold major promise as a novel strategy for reducing cardiac injury from ischemia/reperfusion (Weiss et al. 2003). Our findings suggest that SFA could be a novel candidate for cardioprotection from ischemia/reperfusion injury. As an endogenous substance, SFA could be expected to have minimal, if any, unpredictable side effects. SFA has been used in cardiomyocytes at a high concentration (100 μM), but this concentration is achievable *in vivo*. In our cerebral ischemia model, we administered SFA, at 10 mg/kg i.v. This dose could theoretically lead to approximately 300 μM SFA in the circulating blood. Despite the positive prospect of SFA as a novel

cardioprotective agent, further investigations in animal models are needed to assess the infarct size-limiting effect. Those experiments are currently underway in our laboratory.

ADDENDUM

Selected Abbreviations

SFA = serofendic acid
$\Delta\Psi_m$ = mitochondrial membrane potential
mitoK_{ATP} channel = mitochondrial ATP-sensitive potassium channel
MPTP = mitochondrial permeability transition pore
$[Ca^{2+}]_m$ = mitochondrial matrix calcium
ROS = reactive oxygen species
TMRE = tetramethylrhodamine ethyl ester
5-HD = 5-hydroxydecanoate

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識る 7

Brugada症候群と遺伝子病

－Brugada症候群とQT延長症候群など他の遺伝子病との関連について－

▷*Brugada syndrome and genetic disease*

牧山 武，赤尾昌治（京都大学大学院医学研究科循環器内科）
伊藤英樹，堀江 稔（滋賀医科大学呼吸循環器内科）

Brugada症候群は，1992年，心電図上，右脚ブロック・右側胸部誘導における特徴的なST上昇を示し，心室細動による突然死を生じる症候群としてBrugada兄弟らにより報告された。1998年には，Brugada症候群の家系においてヒト心筋Naチャネルαサブユニットをコードする*SCN5A*遺伝子異常が報告されたが，すでに1995年，遺伝性QT延長症候群3型の原因として同定されていた遺伝子と同一であった。現在まで，この*SCN5A*遺伝子異常によりさまざまな遺伝性不整脈が起こることが報告されている。パッチクランプを用いた変異チャネルの機能解析により，ヒト心筋Naチャネルが機能亢進（電流量が増加）すると，遺伝性QT延長症候群3型を呈し，逆に，機能が低下するとBrugada症候群，進行性心臓伝導欠損（房室ブロック），家族性洞不全症候群を引き起こすことがわかってきた。また，前述の疾患の合併例も報告されオーバーラップ症候群とよばれる。このように，単一の*SCN5A*遺伝子による異常により違う表現型を呈し，しばしばオーバーラップするため，これらは総称して心臓Naチャネル病とよばれる。本稿では，Brugada症候群と他の心臓Naチャネル病について述べる。

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Brugada症候群は，1992年，特に器質的心疾患をもたず，心電図上，右脚ブロック・右側胸部誘導(V_1～V_3)における特徴的なST上昇を示し，心室細動による突然死を生じる疾患としてBrugada兄弟[1]により8症例が系統立てて報告された。1998年，Chenら[2]は，Brugada症候群の家系において，心臓Naチャネルαサブユニットをコードする*SCN5A*遺伝子のヘテロなミスセンス変異，スプライシング異常，一塩基欠失を検出した。その後，培養細胞に変異チャネルを発現させた再構築系においてNa電流を解析するという手法により，Brugada症候群で検出された多くの*SCN5A*遺伝子変異の機能解析がなされ，Na電流量低下(Naチャネルの機能低下：loss of function)が原因であることが示された[3-6]。

この*SCN5A*遺伝子は，すでに1995年，Wangら[7]により，遺伝子連鎖解析，ポジショナルクローニングを用いて遺伝性QT延長症候群3型(LQT3)の原因遺伝子として同定されていた。QT延長症候群(long QT syndrome；LQTS)は，心電図上，QT延長と多彩なT波の形態異常を示し，多形性心室頻拍torsade de pointesによる失神，突然死をきたす。現在，遺伝性LQTSは原因遺伝子により主に6型まで分類され(**表1**)，それぞれ表現型が異なる。LQT1は，水泳などの運動時に失神発作が多くβ遮断薬が第一選択薬となる。LQT2は，精神的ストレスや電話のベルによる驚愕など情動時に発作が起きやすい。LQT3では，失神発作は安静時や夜間の睡眠時に起きやすくβ遮断薬は無効である。Naチャネル異常はこの3型のみであり，頻度は遺伝性QT延長症候群の約10%である[8]。小児では乳幼児突然死症候群(sudden infant death syndrome；SIDS)との関連も報告されている[9]。われわれも新生児でQT延長とtorsade de pointesを認める例(**図1**)にて*SCN5A*変異(N1774D)を検出した。LQT3のパッチクランプ法を用いた電気生理学的機能解析ではNaチャネルが活性化した後，完全に不活化されずわずかな内向き電流が持続し(遅延Na電流)，そのため正味のNa電流量が増加し，活動電位持続時間が延長するというNaチャネルの機能亢進(gain of function)がみられる[10,11]。

その後，1999年，進行性伝導欠損(progressive cardiac conduction defect)の大家系(家系52症例中，有病者15症例)において*SCN5A*遺伝子のスプライシング異常が検出された[12]。進行性伝導欠損は，Lenègre病ともいわれ，His-Purkinje刺激伝導系の進行性障害により，脚ブロック，QRS幅の延長を認め，完全房室ブロックから失神や突然死をきたす。スプライシング異常のアレルからはまったく機能しないチャネル蛋白がつくられ，Brugada症候群と同様にNaチャネルのloss of functionが原因であると考えられた。

近年，家族性洞不全症候群においても*SCN5A*遺伝子変異によるNaチャネルの機能低下が原因となることが報告された[13]。Bensonらは7家系10症例の小児の洞不全症候群患者において*SCN5A*遺伝子をスクリーニングし，それぞれ異なるアレル上の2つの*SCN5A*変異が合併した3症例においてのみ常染色体劣性遺伝形式で洞不全症候群を発症していることを示した。変異チャネルを用いた機能解析では，2つの*SCN5A*変異が合併することにより機能低下の程度が増し，洞結節から心房筋への興奮伝導が障害され洞不全症候群を発症すると考えられた。

表1　遺伝性QT延長症候群の遺伝分子学的分類

常染色体優性 (Romano-Ward症候群)，ヘテロ異常	遺伝子	影響を受けるイオン電流
LQT1	*KCNQ1*	I_{Ks}
LQT2	*KCNH2*	I_{Kr}
LQT3	*SCN5A*	I_{Na}
LQT4	*Ankyrin*-B	Na/Ca exchanger，I_{Na}，$[Ca^{2+}]i$
LQT5	*KCNE1*	I_{Ks}
LQT6	*KCNE2*	I_{Kr}
常染色体劣性(Jervelle & Lange-Nielsen症候群)，ホモ異常，難聴を合併		
JLN1	*KCNQ1*	I_{Ks}
JLN2	*KCNE1*	I_{Ks}

他に，拡張型心筋症や家族性心房細動と*SCN5A*遺伝子異常との関連も報告されている[14,15]。

オーバーラップ症候群

このように*SCN5A*遺伝子異常により，Brugada症候群，遺伝性QT延長症候群3型，進行性伝導欠損，洞不全症候群が生じることが明らかになってきたが，同じ*SCN5A*遺伝子変異でも，単一の症候群でなく，多疾患の表現型を同時に呈するオーバーラップ症候群も少なくない。

(1)Brugada症候群とLQT3

Bezzinaら[16]は，8世代にわたるドイツ人の家系においてBrugada様心電図と徐脈時のQT延長の合併を認め(図2)，遺伝子解析にて*SCN5A*の1アミノ酸挿入変異(1795insD)を検出した。この変異チャネルの機能解析[17]では，内向き遅延Na電流というLQT3でみられるgain of function(図3a)と，不活性化の膜電位依存性が過分極方向へシフトするというBrugada症候群でみられるloss of functionの機能変化(図3b)を同時に併せもち，そのためQT延長とBrugada心電図を併せもつと考えられた。この家系では，両疾患を同時に合併する症例もあれば，片方のみの場合もあり，表現型の発症には他の環境因子や遺伝的因子の影響が示唆される。

(2)Brugada症候群と伝導障害(洞不全症候群も含む)

Kyndtら[18]は，フランス人の家系45症例中，13症例にG1406Rのヘテロ*SCN5A*変異を検出した。変異キャリア中，4症例にBrugada症候群，7症例に心臓伝導障害を認めた。パッチクランプを用いた機能解析ではこの変異チャネルはまったく電流を形成しない重度の機能低下を示した。また，Brugada症候群と洞停止[19]，あるいは洞不全症候群[20]の合併例も報告されて

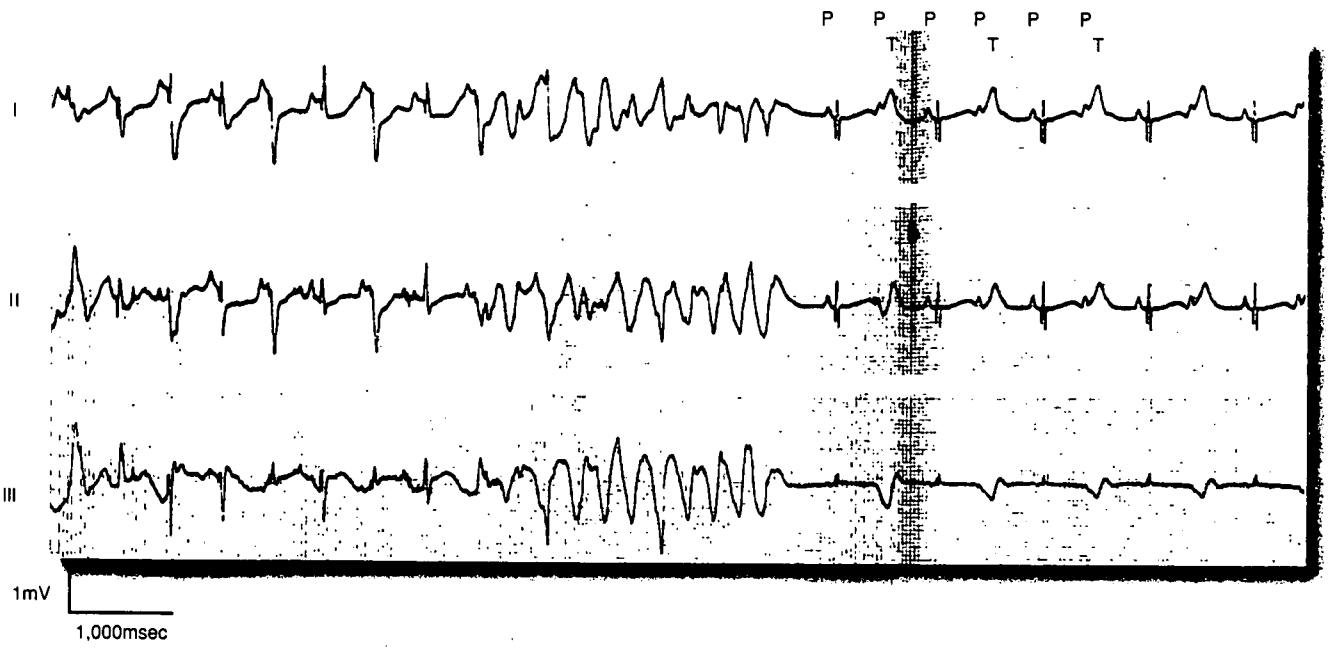

図1 ニアミス新生児突然死症候群の心電図
生後1日目の男児。QTc=0.72と著明に延長し，torsade de pointesを認める。T波(T)が次のP波(P)より遅れ，2：1房室ブロックをきたしている。*SCN5A*遺伝子変異(N1774D)を認めた。

いる。

われわれは，最近，日本のBrugada症候群における徐脈性不整脈(房室ブロックまたは洞不全症候群)の合併について報告した[21]。多施設からの日本人Brugada症候群38症例を対象とし，*SCN5A*遺伝子スクリーニングを行ったところ，38症例中，4症例(4家系)においてヘテロ*SCN5A*遺伝子変異(T187I，D356N，K1578fs/52，R1623X)を検出した(**図4**)。興味深いことに，この4症例とも徐脈性不整脈(3症例が洞不全症候群，D356Nが完全房室ブロック)を合併し(**図5**)，パッチクランプ法を用いた電気生理学的機能解析では，4変異ともまったくNa電流を形成しないnon-functionalな遺伝子変異であった(**図6**)。また，T187I(**図5a**)，K1578fs/52(**図5c**)の家系において，洞不全症候群によるペースメーカー植え込みの家族歴を認めた。発端者の徐脈性不整脈の合併に注目

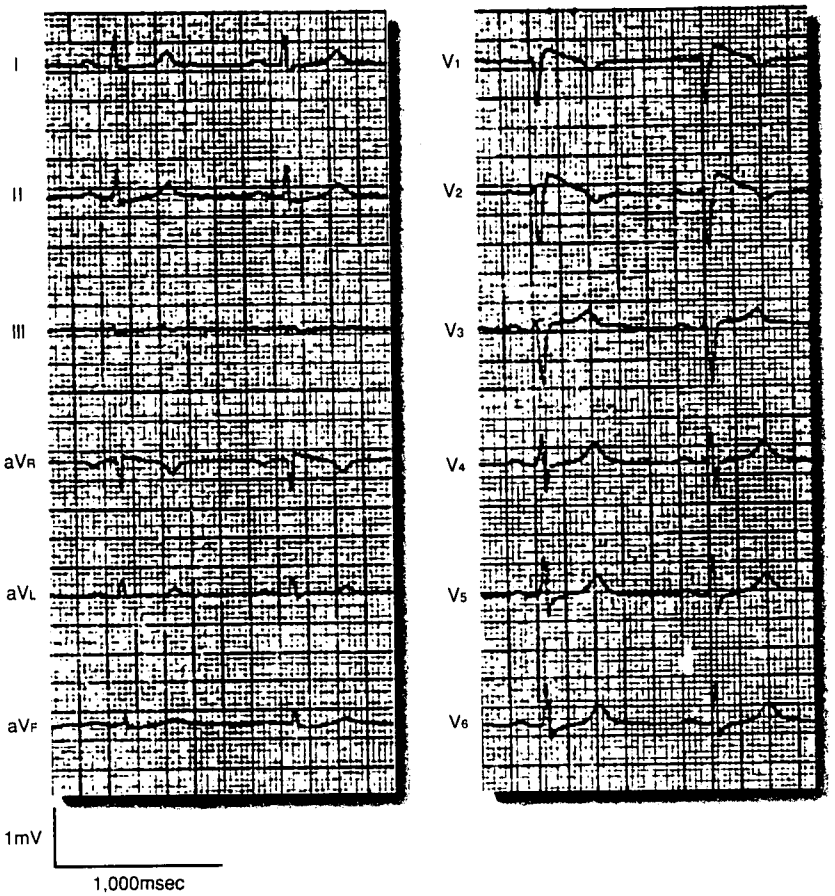

図2　1795insDの心電図(文献16より改変引用)
Brugada様心電図と軽度QT延長(QTc：0.465)を認める。

すると，*SCN5A*遺伝子異常を認めるBrugada症候群では徐脈性不整脈の合併が4/4と，遺伝子異常を認めないBrugada症候群(2/34)に比べ有意に多いことがわかった。この研究より*SCN5A*遺伝子異常を認めるBrugada症候群患者では，高率に徐脈性不整脈を合併することが示唆された。遺伝子解析にて*SCN5A*遺伝子異常がみつかりnon-functionalな変異の場合には，徐脈性不整脈の合併に注意すべきであると考えられた。

R1623X(**図5d**)ナンセンス変異は，Bensonら[13]により，別の*SCN5A*変異(T220I)が合併することにより，常染

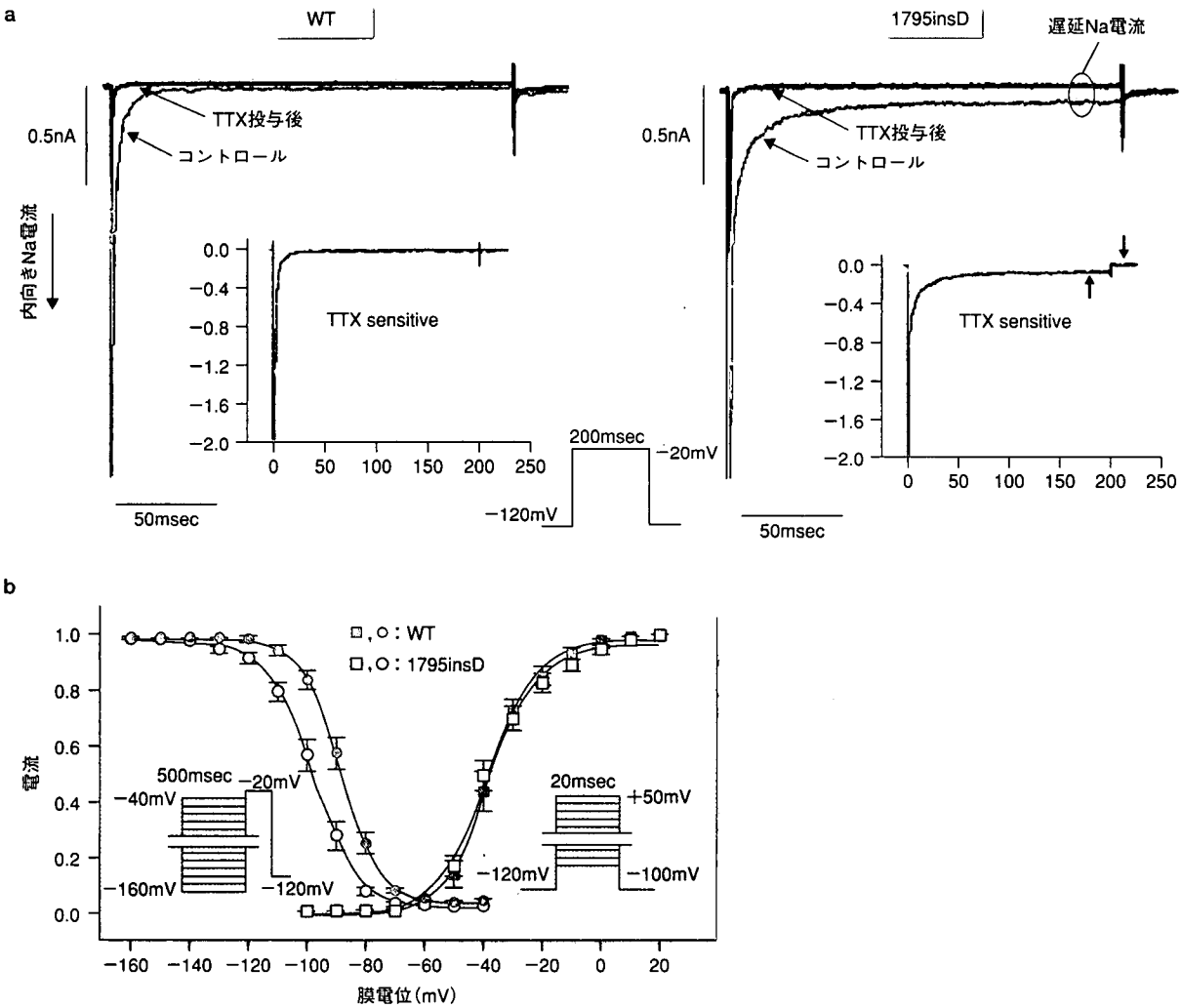

図3 Brugada症候群とQT延長症候群の合併例にみられた変異チャネル(1795insD)の機能解析
(文献17より改変引用)

a：ヒト胎児腎由来培養細胞(HEK293 cell)に正常(WT)，および，変異チャネル(1795insD)を発現させ記録したNa電流。正常チャネルでは，脱分極により内向き電流が流れた後ほぼ完全に不活性化されるが，変異チャネルでは，不活性化障害のため遅延Na電流がみられる。全体としてNa電流量は増加するため，gain of functionとなる。図内右下は，テトロドトキシン(TTX：Naチャネル遮断薬)を用いてサブトラクションした電流を示す。

b：変異チャネルでは，不活性化の膜電位依存性は過分極方向へ偏位し，Naチャネルのloss of functionを認める。

色体劣性遺伝形式にて，洞不全症候群を呈したと報告されている。彼らの家系でR1623Xのみをもつ症例は，1度房室ブロックをきたすのみであった。しかし，われわれの症例は，R1623X単独でペースメーカー植え込み後，夜間心室細動をきたした典型的なBrugada症候群を発症しており，同じ*SCN5A*変異をもっていても表現型が異なる可能性が示唆された。

また，K1578fs/52(**図5c**)の症例は，家族性洞不全症候群(家系内に4人ペースメーカー植え込み)としてフォローされていたが，Naチャネル遮断薬負荷でST上昇を示し，電気生理検査にて心室頻拍が誘発された。このように家族性洞不全症候群をみかけた場合，*SCN5A*遺伝子異常の可能性を考え，Brugada症候群の病態の有無にも注意が必要な場合があると思われる。

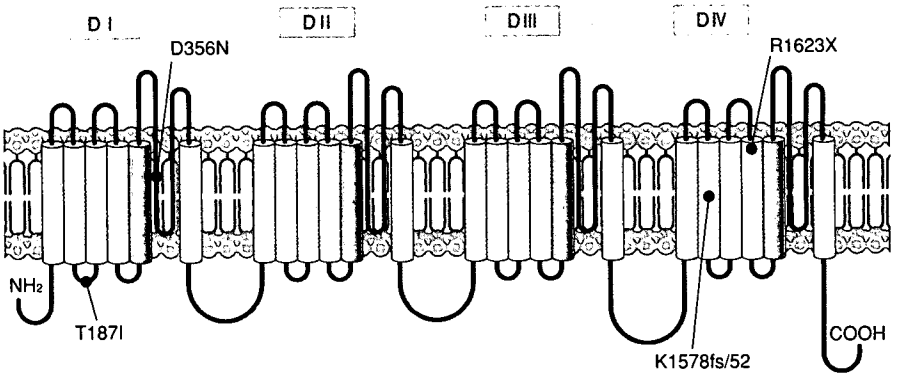

図4　心臓Naチャネルの模式図
(文献21より改変引用)
われわれが検出した4変異の位置を示す(T187I，D356N：ミスセンス変異，K1578fs/52：フレームシフト変異，R1623X：ナンセンス変異)。心臓Naチャネルαサブユニットは，4つのドメインからなり，それぞれ6つの膜貫通領域をもつ。

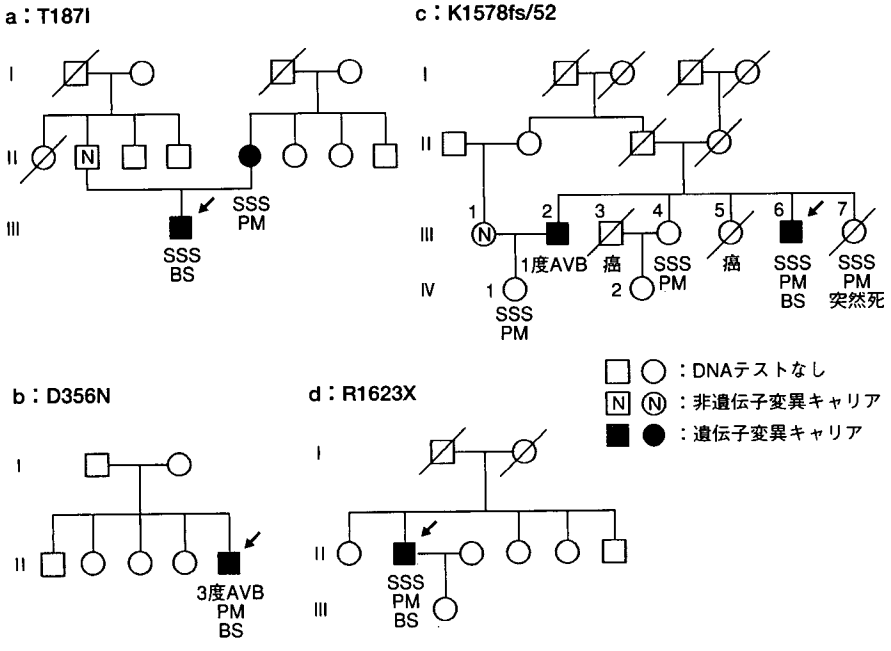

図5　家系図(文献21より改変引用)
丸印は女性，四角は男性。矢印は発端者を示す。斜線は死亡している家族で，遺伝子変異キャリアは赤塗りで示す。AVB：atrioventricular block，BS：Brugada syndrome，PM：patients with a pacemaker，SSS：sick sinus syndrome。

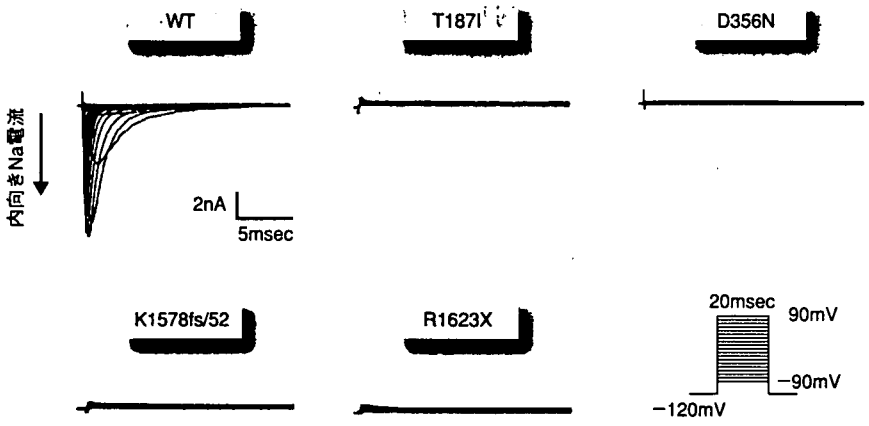

図6　変異チャネルの機能解析(文献21より改変引用)
ヒト胎児腎由来培養細胞に正常(WT)，および，変異チャネルをβサブユニットと共発現させ，ホールセル・パッチクランプ法を用いてNa電流を記録した。4つの変異チャネルは，まったくNa電流を形成せず，complete loss of functionであった。

最後に

現在，*SCN5A*遺伝子異常によりいくつかの不整脈疾患が生じることがわかっており，それらのオーバーラップも少なくない。これら全体を心臓Naチャネル病という概念(図7)で捉えることができる。心臓Naチャネル病では，原因遺伝子が同じでも変異が違えば，Naチャネルの機能が変化し，異なる病態を発症するという表現型の多様性が認められる。また，同じ変異でも個人で症状が異なる場合があり，他の環境因子，遺伝的因子の影響も考え

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られるが，まだ不明な点が多い。Brugada症候群は心臓Naチャネル病の1つであり，他の表現型をしばしば併せもつ。Brugada症候群の診療においては，他の表現型（LQT3，伝導障害）の合併も考慮に入れたフォローが必要であると考えられる。

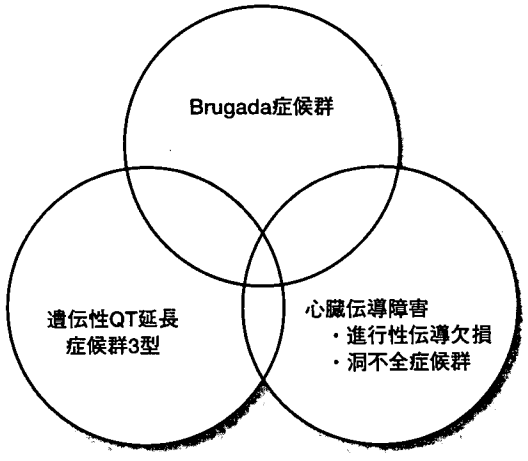

図7　心臓Naチャネル病の概念図

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The Effects of Long-term Smoking on Endothelial Nitric Oxide Synthase mRNA Expression in Human Platelets as Detected With Real-time Quantitative RT-PCR

Yukio Shimasaki, MD,* Yoshihiko Saito, MD,† Michihiro Yoshimura, MD,* Shigeki Kamitani, MS,‡ Yoshihiro Miyamoto, MD,‡ Izuru Masuda, MD,‡ Masafumi Nakayama, MD,* Yuji Mizuno, MD,§ Hisao Ogawa, MD,* Hirofumi Yasue, MD,§ and Kazuwa Nakao, MD‡

*Department of Cardiovascular Medicine, Kumamoto University Graduate School of Medical Sciences, Kumamoto; †First Department of Internal Medicine, Nara Medical University, Nara; ‡Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto; and §Division of Cardiology, Kumamoto Kinou Hospital, Kumamoto Aging Research Institute, Kumamoto, Japan

Summary: Endothelium-derived nitric oxide (NO) plays an important role in the prevention of platelet aggregation and adhesion to the vascular wall. Endothelial nitric oxide synthase (eNOS) and L-arginine/NO pathway are both present in human platelets. Platelet-derived NO inhibits excessive activation and aggregation of platelets. However, the expression level of the eNOS gene in human platelets has yet to be elucidated. The current study investigates the individual expression level of platelet eNOS mRNA using the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) detection method. eNOS mRNA expression was examined in platelets isolated from 50 subjects: 11 male smokers, 15 male nonsmokers, and 24 female nonsmokers. After extraction of platelet total RNA, eNOS (target) and GAPDH (internal control) mRNA expression levels were quantitated using real-time RT-PCR. The expression levels of eNOS mRNA (relative copy numbers) were significantly lower in male smokers (59±17) than in male nonsmokers (195±71, $P < .03$), and higher in female nonsmokers (285±60) than in the male nonsmokers (195±71, $P < .03$). By multiple linear regression analysis, cigarette smoking ($P = .008$) and diabetes mellitus ($P = .047$) were found to be significantly negative predictors, and antioxidant (vitamin E) treatment ($P = .01$) was a significantly positive predictor of platelet eNOS mRNA expression. Age, other medications, and other risk factors for coronary artery disease were not significant. Using this method, eNOS mRNA abundance in human platelets was detected and quantitated in real-time. The intraplatelet eNOS mRNA expression levels were significantly decreased in cigarette smokers. Low platelet NO synthesis in smokers may result in the augmentation of platelet aggregation and thrombus formation, developing into acute coronary syndromes.

Key Words: Platelets—Nitric oxide synthase—Cigarette smoking—Risk factors.

Nitric oxide (NO) is synthesized from the guanidino nitrogen atom(s) of L-arginine through a reaction catalyzed by three isoforms of nitric oxide synthase (NOS). The third isoform, endothelial NOS (eNOS or NOS-III), a 133-kDa membrane-bound protein, is constitutively expressed primarily in endothelial cells and is considered critical for physiologic endothelial function and cardiovascular homeostasis (1–3). However, recent reports have shown that the level of eNOS gene expression can be altered by a growing number of different stimuli, including shear stress, estrogens, lysophosphatidylcholine, tumor necrosis factor (TNF)-α, lipopolysaccharide, hypoxia, and so on (4). Bioactive NO produced by eNOS in vascular endothelium plays a key role in the regulation of vascular tone. Endothelium-derived NO has been shown to

This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Tokyo, Japan; Research Grant from TMFC (Tanabe Medical Frontier Conference), Tokyo, Japan; and Ueharn Memorial Foundation, Tokyo, Japan.

Address correspondence and reprint requests to Yukio Shimasaki, MD, Department of Cardiovascular Medicine, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto City, 860-8556, Japan; e-mail: yuki7nao@mocha.oen.ne.jp.

Clinical and Applied Thrombosis/Hemostasis
Vol. 13, No. 1, January 2007 43-51
DOI: 10.1177/1076029606296402