have to be tested in humans. Will their efficacy on bone in humans be the same as in mice? Will they have beneficial effects on non-skeletal tissues? Will reproductive tissues, such as the uterus and breast, be totally non-responsive? The answers to these and other questions will ultimately determine the fate of mechanism-specific ligands. Regardless of the outcome, however, few would disagree that a novel therapeutic approach has been defined, and the scientific journey that beckons will be fascinating both for the investigators and for the rest of us, with patients perhaps being the ultimate beneficiaries.

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From nitric oxide to endothelial cytosolic Ca^{2+}: a negative feedback control

Xiaoqiang Yao and Yu Huang

Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Hong Kong, China

Nitric oxide production is stimulated by an increase of the concentration of cytosolic Ca^{2+} in vascular endothelial cells. Recent evidence suggests that nitric oxide and cGMP might attenuate Ca^{2+} influx and, at the same time, initiate a Ca^{2+} removal mechanism, thereby decreasing the intracellular concentration of endothelial Ca^{2+} in a negative feedback fashion. Such a negative feedback mechanism could serve to protect the endothelial cells from the detrimental effects of excessive nitric oxide and Ca^{2+}.

The discovery of nitric oxide (NO) as a vasoactive substance is one of the great advances in vascular biology. It is now generally accepted that physical stimuli and numerous blood-borne chemicals can elicit an increase of the intracellular concentration of Ca^{2+} {$[Ca^{2+}]_i$} in endothelial cells, which in turn activates endothelial NO synthase (eNOS) and phospholipase A_2, leading to the production and release of NO and other vasoactive substances [1]. In addition to its role in the regulation of vascular tone, endothelial Ca^{2+} signaling influences diverse cellular processes such as gene expression, exocytosis and cell proliferation.

The effective regulation of vascular tone by NO and $[Ca^{2+}]_i$ requires efficient mechanisms to tightly control their levels. Typically, physical or chemical stimuli elicit a transient increase of $[Ca^{2+}]_i$, which is quickly followed by a decay phase in which $[Ca^{2+}]_i$ decreases and approaches the basal level as a result of the attenuation of Ca^{2+} influx and the activation of Ca^{2+} removal mechanisms. However, what are the signals and/or mechanisms that switch on the Ca^{2+} removal pathways and switch off the Ca^{2+} influx pathways when $[Ca^{2+}]_i$ is high in endothelial cells? Part of the answer lies with a direct action of $[Ca^{2+}]_i$. When $[Ca^{2+}]_i$ reaches a certain level, Ca^{2+} binds to channels that mediate an influx of Ca^{2+} and elicits rapid inactivation of these channels within tens of milliseconds [2]. In general, however, the decrease of $[Ca^{2+}]_i$ follows a slower time-course than does the rapid inactivation of these channels [2]. Recent evidence suggests that endothelial NO, the production of which is stimulated by an increased $[Ca^{2+}]_i$, can reduce the endothelial $[Ca^{2+}]_i$ in a negative feedback fashion (Fig. 1).

Effects of NO on SERCA

The major subtypes of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in vascular endothelial cells are SERCA2b and SERCA3 [3]. When $[Ca^{2+}]_i$ rises, the activity of SERCA is increased to clear cytosolic Ca^{2+} into the endoplasmic reticulum, contributing to the decay phase of the transient increase of $[Ca^{2+}]_i$ that is induced by physical or chemical stimuli [4]. In the absence of extracellular Ca^{2+}, the NO donor sodium nitroprusside (SNP) accelerates the decay phase, resulting in a faster return of $[Ca^{2+}]_i$ to the basal level [5]. Furthermore, prolonged incubation of endothelial cells with SNP results

Corresponding author: Xiaoqiang Yao (yao2068@cuhk.edu.hk).

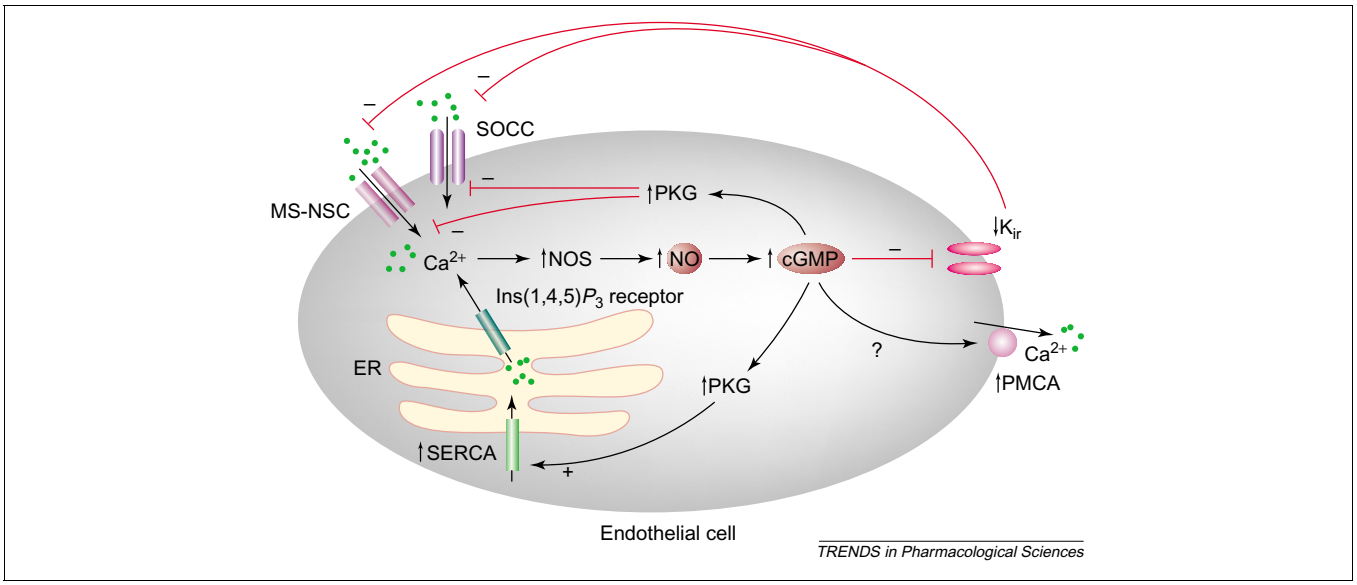

Fig. 1. A schematic drawing of the negative feedback pathways by which nitric oxide (NO) and cGMP might reduce the intracellular concentration of cytosolic Ca^{2+} in endothelial cells. A rise in the concentration of endothelial cytosolic Ca^{2+} can result from Ca^{2+} release from intracellular stores [e.g. endoplasmic reticulum (ER)] via inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] receptors and from Ca^{2+} influx through store-operated Ca^{2+} channels (SOCCs) and/or mechanosensitive Ca^{2+}-permeable nonselective cation channels (MS-NSCs) on the plasma membrane. An increased concentration of cytosolic Ca^{2+} stimulates the activation of nitric oxide synthase (NOS), which leads to the production of NO and cGMP. Elevated cGMP can decrease the intracellular concentration of Ca^{2+} in a negative feedback mechanism via several pathways. Thus, cGMP activates protein kinase G (PKG), leading to the inhibition of the influx of Ca^{2+} through SOCCs and/or MS-NSCs and the enhanced uptake of Ca^{2+} into intracellular stores by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). cGMP also directly inhibits inward-rectifying K^+ channels (K_{ir}), decreasing the driving force for Ca^{2+} influx. cGMP might also stimulate the plasma membrane Ca^{2+} pump (PMCA), leading to increased extrusion of Ca^{2+} from the cell. Symbols: +, activation; −, inhibition.

in enhanced Ca^{2+} loading into the endoplasmic reticulum, as revealed by direct measurements of Ca^{2+} in intracellular stores using mag-fura-2 [6]. These data suggest that NO stimulates the activity of SERCA in endothelial cells, although the detailed molecular mechanism of this action is still unclear. Nevertheless, studies in smooth muscle cells might provide some clues. In vascular smooth muscle cells, the background activity of SERCA is inhibited by the integral sarcoplasmic reticulum protein phospholamban. NO, through the cGMP–protein kinase G (PKG) pathway, causes phosphorylation of phospholamban, thereby relieving inhibition of SERCA and enhancing SERCA activity [7]. Because guanylyl cyclase, PKG and phospholamban are all present in vascular endothelial cells [5,8], it is possible that NO modulates endothelial SERCA in a similar way.

Effects of NO and cGMP on Ca^{2+} influx

As in many other non-excitable cells, the predominant pathway by which Ca^{2+} enters vascular endothelial cells is through store-operated Ca^{2+} channels (SOCCs) on the plasma membrane (Fig. 1) [2]. Indeed, diverse vasoactive agonists such as bradykinin, ATP and histamine are thought to stimulate Ca^{2+} influx via depletion of intracellular Ca^{2+} stores. SERCA inhibitors such as thapsigargin can also stimulate store-operated Ca^{2+} influx by blocking the uptake of Ca^{2+} into intracellular stores and causing subsequent store depletion.

Several lines of evidence suggest that the NO–cGMP–PKG pathway downregulates store-operated Ca^{2+} influx. First, the store-operated Mn^{2+} influx elicited by ATP or thapsigargin is greatly attenuated by SNP [6] and brain-type natriuretic peptide [5], which stimulate the production of cGMP. The effect of SNP is due to NO because it can be mimicked by another NO donor, S-nitroso-N-acetylpenicillamine (SNAP), and is absent in the presence of the NO scavenger hemoglobin. Second, cGMP inhibits store-operated Ca^{2+} influx [6,9], and patch-clamp recording shows that cGMP inhibits a store-operated Ca^{2+}-permeable channel [9]. The inhibitory effect of cGMP on both Ca^{2+} influx and the activity of Ca^{2+}-permeable channels is abolished in the presence of the PKG inhibitors KT5823 and H8 (see Chemical names), suggesting that the effect of cGMP is mediated through PKG [9]. Third, interesting studies performed in vascular smooth muscle cells and platelets by Cohen *et al.* show that the NO–cGMP–PKG pathway might reduce Ca^{2+} influx by stimulating SERCA-mediated store refilling. Replenishment of intracellular stores subsequently attenuates the store-operated Ca^{2+} influx pathway [10,11]. This finding might be of general importance because it is consistent with the concept that the filling status of intracellular Ca^{2+} stores is a key signal for the control of store-operated Ca^{2+} influx [2,10,11]. However, this mechanism cannot account for the attenuating effect of NO and cGMP on store-operated Ca^{2+} influx observed by Kwan *et al.* [9] and Delkova *et al.* [6] because in the latter two cases SERCA was already inactivated by pre-incubation with thapsigargin. Thus, the NO–cGMP–PKG pathway might act through multiple pathways to inhibit SOCCs.

In addition to the store-operated Ca^{2+} influx pathway, vascular endothelial cells have a shear stress-sensitive Ca^{2+} influx pathway. A mechanosensitive, Ca^{2+}-permeable, nonselective cation channel (MS-NSC) is present in

Chemical names

H8: N-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide

endothelial cells and it is speculated that this channel might serve as a mechanosensor, transforming the mechanical force generated by hemodynamic shear stress into chemical changes in endothelial cytosolic Ca^{2+} [1]. Recently, the activity of a similar channel was recorded in rat aortic endothelial cells and was shown to be attenuated by PKG [12]. PKG not only inhibits the channel but also greatly reduces shear stress-induced Ca^{2+} influx, suggesting that this channel is at least one of the candidates responsible for shear stress-induced Ca^{2+} influx [12–14]. In terms of single-channel conductance, ion selectivity profile and PKG inhibition, this mechanosensitive channel appears to be similar to the store-operated Ca^{2+}-permeable channel in endothelial cells. A recent study also showed that store-operated and mechanosensitive Ca^{2+} influx pathways are inter-connected because depletion of intracellular Ca^{2+} stores causes endothelial Ca^{2+} influx to become more sensitive to shear stress [15]. However, no direct evidence is available to determine whether these two channels are completely different channels, each with a distinct molecular identity, or whether the channels belong to the same class of channels. One suggestion is that these might belong to the transient receptor potential (TRP) channel family. Vascular endothelial cells contain numerous TRP channels, many of which are suggested to be involved in store-operated Ca^{2+} influx [1,16]. Indeed, compelling evidence suggests that TRPC1 and TRPC4 have an important role in store-operated Ca^{2+} influx in vascular endothelial cells [16]. Furthermore, TRPV4 might be involved in mechanosensing [16]. However, it is not known whether the activity of these TRP channels is downregulated by the NO–cGMP–PKG pathway.

The NO–cGMP pathway can also modulate endothelial K^+ channels (Fig. 1). Inward-rectifying K^+ channels (K_{ir}) represent one of the important channel classes that modulates the membrane potential of endothelial cells [1]. Whole-cell patch-clamp recording by Shimoda *et al.* showed an inhibitory effect of cGMP on the K_{ir} channel currents in bovine pulmonary endothelial cells. The inhibition is due to the direct action of cGMP on K_{ir} channels, independently of PKG [17]. Inhibition of K_{ir} channels should result in membrane depolarization, which decreases the driving force for Ca^{2+} influx.

Effects of NO and cGMP on PMCAs

The plasma membrane Ca^{2+} pumps (PMCAs) are encoded by four distinct genes. PMCA isoform 1 is expressed in vascular endothelial cells [18]. There are conflicting reports regarding the effect of NO and cGMP on PMCAs in vascular endothelial cells [5,6,19]. For example, Chen *et al.* [19] have demonstrated that NO reduces $[Ca^{2+}]_i$ by stimulating PMCAs in cultured bovine aortic endothelial cells whereas two other reports [5,6] showed an inhibitory effect of the NO–cGMP pathway on PMCAs. Further research is needed to produce a more definitive answer.

Conflicting studies

The reports regarding the effect of NO and cGMP on endothelial $[Ca^{2+}]_i$ are not always consistent. For example, in addition to the conflicting reports of the NO effect on PMCAs, Chen *et al.* [19] have shown that NO potentiates Ca^{2+} influx but has no effect on SERCA in cultured bovine endothelial cells. One possible reason for inconsistency could be the instability of PKG and guanylyl cyclase in cell culture conditions [20]. Loss of enzyme activity would impair the NO–cGMP–PKG pathway and therefore the effect of this pathway might be overlooked.

Concluding remarks

Endothelial NO production is stimulated by a rise in $[Ca^{2+}]_i$. Accumulating evidence now indicates that elevated NO and cGMP can attenuate Ca^{2+} influx and, at the same time, initiate Ca^{2+} removal mechanisms, thereby decreasing the endothelial $[Ca^{2+}]_i$ in a negative feedback fashion. This negative feedback mechanism, which might also operate in other cell types such as vascular smooth muscle cells, platelet, pancreatic cells and HEK293 cells [7,10,11,21,22], might protect endothelial cells from the detrimental effects caused by excessive NO and $[Ca^{2+}]_i$ [23].

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Cannabis and alcohol – a close friendship

Raphael Mechoulam[1] and Linda Parker[2]

[1]Hebrew University Medical Faculty, Pharmacy School Department of Medicinal Chemistry and Natural Products, Jerusalem 91120, Israel
[2]Wilfrid Laurier University Department of Psychology Waterloo, Ontario N2L 3C5, Canada

Cannabinoids and alcohol activate the same reward pathways, and the cannabinoid CB_1 receptor system plays an important role in regulating the positive reinforcing properties of alcohol. Indeed, both cannabinoids and alcohol cause the release of dopamine in the nucleus accumbens. Recent research suggests that ethanol preference, which is dependent on CB_1 receptors, is higher in young mice than in old mice, and higher in female mice than in male mice.

Cannabis and alcohol have seldom gone hand-in-hand in societies where both are consumed. Franz Rosenthal, the eminent Yale orientalist, has translated from Arabic a long rhymed debate between imaginary pro-hashish and pro-wine protagonists, written by a Syrian poet in the 13th century [1].

The pro-hashish party:
No! Unlike wine it is a gift
It is the spirit pure
Free as it confines from worry
Only the elect may taste it.

The pro-wine party:
Unlike hashish, its qualities are useful
Speak out! Count and describe wine's many meanings.

However, the research groups of Kunos and Hungund have now shown that there is a close link between cannabis and alcohol [2,3]. They have found that cannabinoids and alcohol activate the same reward pathways and that the CB_1 receptor plays an important role in regulating the positive reinforcing properties of alcohol.

Feeding and alcohol – the effect of a cannabinoid receptor antagonist

The Kunos group at the National Institute on Alcohol Abuse and Alcoholism started from the premise that alcoholism is an appetitive disorder. It is well established that endogenous cannabinoids (endocannabinoids) are positive modulators of food intake [4]. Thus, anandamide, the first endocannabinoid to be identified, enhances appetite in mice [5,6], and mice that are deficient in cannabinoid CB_1 receptors ($CB_1^{-/-}$ mice) eat less than their wild-type littermates ($CB_1^{+/+}$ mice). The CB_1 receptor antagonist SR141716A (see Chemical names) reduces hunger and is in clinical trials as an anti-obesity drug [7]. SR141716A also reduces voluntary alcohol intake in rodent models (see below). Thus, Kunos and colleagues investigated the effect of knockout of CB_1 receptors on alcohol consumption. Young wild-type C57Black/6J mice (a strain known to have high preference for alcohol) consumed significantly more alcohol than did $CB_1^{-/-}$ mice. SR141716A caused a significant reduction in the consumption of alcohol in C57Black/6J mice but caused no change in $CB_1^{-/-}$ mice [2].

Gender differences in alcohol consumption

Hungund *et al.* reported results that were essentially identical to those of the Kunos group, and in addition found that there was a gender difference in alcohol

Chemical names

CP55940:(−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
SR141716A:N-piperidono-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide

Corresponding author: Raphael Mechoulam (Mechou@cc.huji.ac.il).