**Gi-protein-coupled β1-adrenergic receptor: Re-understanding the selectivity of β1-adrenergic receptor to G protein**

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Review

Gi-protein-coupled β₁-adrenergic receptor: re-understanding the selectivity of β₁-adrenergic receptor to G protein

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Abstract
β₁-adrenergic receptor (β₁-AR), a member in the family of G-protein-coupled receptors, is a transmembrane receptor of great significance in the heart. Physiologically, catecholamines activate β₁-AR to initiate a positive chronotropic, inotropic, and dromotropic change. It is believed that β₁-AR couples to Gs protein and transmits the signal through second messenger cAMP. However, increasing research shows that β₁-AR can also bind with Gi...
protein in addition to Gs. When β1-AR-Gi is biasedly activated, cardioprotective effects are introduced by the activated cGMP-protein kinase G (PKG) pathway and the transactivation of epidermal growth factor receptor (EGFR) pathway. The discovery of β1-AR-Gi signaling makes us reconsider the selectivity of G protein with regard to β1-AR, which also provides new ideas for the treatment of heart diseases. This review summarizes the discovery of β1-AR-Gi pathway, including the evidence that supports β1-AR’s capability to couple Gi, details of the transduction process and functions of the β1-AR-Gi signaling pathway.

Keywords: β1-adrenergic receptor, Gi protein, G protein switch, signal transduction, cardioprotective effect

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of membrane proteins in the human body, which transmit extracellular information into the intracellular signaling [1]. β1 Adrenergic receptor (β1-AR) is a kind of GPCRs widely distributed in the cardiovascular system. In the heart, β1-AR accounts for approximately 80% of β-adrenergic receptors, with the remaining 20% belonging to β2 adrenergic receptor (β2-AR) and β3 adrenergic receptor (β3-AR) respectively [2, 3]. Under physiological conditions, β1-AR is a critical receptor protein regulating cardiac function. β1-AR can be activated by catecholamines, such as norepinephrine (NE) and epinephrine, to play the role of positive inotropic, positive chronotropic and positive dromotropic action to meet the needs of the body’s fight-or-flight response [3]. In pathological states, β1-AR is also the target of drug therapy for heart diseases [4]. Beta blockers, the inhibitor of β1-AR such as propranolol, metoprolol and carvedilol, can be used for the treatment of heart failure, coronary heart disease, atrial fibrillation, etc. [5-7].

G protein, a guanine-nucleotide-binding protein, is a trimeric complex comprising subunits of Ga, Gβ, and Gγ. According to the variations of the α subunits, G protein can be divided into Gs, Gi, Gq/11, and G12/13 [8-10]. Traditionally, β1-AR is regarded to couple only Gs [11-13]. Activated β1-AR binds to the Gs protein and induces the synthesis of the second messenger cyclic adenosine monophosphate (cAMP) catalyzed by adenylate cyclase (AC),
which phosphorylates downstream proteins through protein kinase A (PKA) to complete signal transduction [14]. Meanwhile, after Gs binds to activated receptor, Gβγ subunit dissociates from Gas subunit and G protein coupled receptor kinase (GRK) is recruited to catalyze receptor phosphorylation [8, 15]. GTP binding induces the separation of Gs from the receptor [8].

Yet, increasing studies have shown that resembling β2-AR, β1-AR can also couple Gi protein in addition to Gs [12, 16]. Recent research has shown that carvedilol biasedly activates the β1-AR-Gi pathway and exerts cardioprotective effects through cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) pathway [17]. Moreover, structural analysis of the β1-AR-Gi complex also underlies a solid foundation for research of the complex [10]. This article uses the beginning and end of β1-AR-Gi signaling as a clue, reviewing the course of the β1-AR-Gi pathway discovery from the very evidence of β1-AR coupling Gi to the process and function of β1-AR-Gi signal transduction, while looking forward to the future β1-AR-Gi research directions.

**Evidence for Gi Coupling β1-AR**

*Early exploration of Gi coupled β1-AR*

In 2002, researchers found that β1-AR has the ability to couple Gi by using neonatal myocytes from β1-AR knockout (KO), β2-AR KO, and β1/2-AR KO mice [12]. However, this feature is usually not manifested due to the existence of PDZ domain binding motif at the C-terminal of β1-AR and PDZ domain-containing scaffolding proteins, through which the coupling of β1-AR and Gi is limited. PDZ domain binding motif mediates specific protein reactions and is, in essence, a protein module with a conserved structure [18]. In this study, the researchers mutated the mouse β1-AR PDZ domain binding motif ~ESKV~ into ~EAAA~ to form the mutant β1-AR (β1-AR-PDZ). Subsequently, HA-labeled mouse wild-type β1-AR or β1-AR-PDZ was transfected into β1/2-AR KO mice neonatal myocytes, and their contraction rate was recorded. The results demonstrated that the contraction rate of isoproterenol (ISO) was first increased and then decreased below the basic level, showing a biphasic contraction rate response, which was consistent with the results shown in β1-AR KO mice neonatal myocytes expressing β2-AR. All of above phenomenon can be blocked by Gi inhibitor pertussis toxin (PTX).
β2-AR is another β-adrenergic receptor in cardiomyocytes that couples Gs and Gi [19, 20]. Additionally, β2-AR KO myocytes were treated with a membrane permeable polypeptide Tag-β1PDZ (466GRQGFSSESKCOO~ of β1-AR) that mimics the binding mode of β1-AR PDZ domain, and ISO stimulation also caused a biphasic contraction response. This effect was inhibited by PTX as well [12]. In general, the above series of results suggest that β1-AR has the potential to couple the Gi protein, but the presence of PDZ restricts the binding of β1-AR to Gi.

In 2004, Lefkowitz’s group [21] showed that the physiological ligand of β1-AR, NE, can also active the β1-AR-Gi pathway. It was found that in Chinese hamster ovary (CHO) cells, when overexpressed β1-AR was stimulated by NE, ERK activity was increased significantly, which could be completely blocked by PTX.

The clarity of direct evidence
In 2017, researchers directly validated Gαi binding to β1-AR when stimulated by carvedilol using the proximity ligation assay (PLA) and co-immunoprecipitation (CO-IP) [16]. Carvedilol is a β-blocker that can biasedly activate β1-AR-β-arrestin signaling [22]. In HEK293 cells overexpressing the Flag-β1-AR, with the increase of carvedilol concentration (10^-9–10^-5 M), the amount of Gi coupling β1-AR gradually increased in a concentration-dependent manner. However, ISO did not trigger β1-AR to Gi binding even in the concentration range of 10^-9 M to 10^-5 M. Forby, the β1-AR-Gi interaction was detected after treating HEK293 cells with 10^-5 M carvedilol for 5 minutes. This is, in fact, time-dependent, as more Gi is bound to β1-AR in a prolonged stimulation time (30 minutes) [16]. The binding of β1-AR to Gi, detected by either PLA or CO-IP experiments, could be blocked by PTX [16], which confirmed that carvedilol is a β1-AR-Gi-biased ligand and promotes the binding in concentration-dependent and time-dependent manners.

Analysis of the structure and function of β1-AR-Gi
In 2021, Xiang’s group [17] found that carvedilol repaired the heart function damage in mice fed with high-fat diet, and, correspondingly, that the protective
effect of carvedilol was reversed by PTX. Apart from further evidence that Gi couples β₁-AR, this study also confirmed the cardioprotective effect of β₁-AR-Gi activation in animal models of cardiac dysfunction. Almost simultaneously, Huang’s group [10] analyzed the structure of the ISO-β₁-AR-Gi complex by cryo-electron microscopy with a resolution of 3.0 Å. Together, these results suggest that Gi can couple β₁-AR in response to agonist or blocker stimulation.

But still, some confusions need to be clarified in order to put us in the picture. (1) How much influence does PDZ domain binding motif have on β₁-AR-Gi binding? The PDZ domain binding motif is one of the reasons, but not the only one that is preventing the binding of β₁-AR to Gi. This is because the PDZ is located at the C-terminal of the receptor. It has been demonstrated that the C-terminal alone is not sufficient to determine the binding of β₁-AR to Gi [16]. Results from earlier research validated that it is the presence of β₁-AR C-terminal PDZ domain binding motif that prevents ISO to activate β₁-AR-Gi combination [12, 16]. Subsequent studies demonstrated that β₁-AR can still bind to Gi even in the absence of external intervention on β₁-AR PDZ domain binding motif stimulated by carvedilol [16, 17]. Although the ligand used in the protein purification of β₁-AR-Gi complex is ISO, there is no PDZ domain-containing scaffolding proteins under the experimental conditions [10]. It suggests that different drugs may vary in modes that cause β₁-AR-Gi binding. Apparently, while carvedilol causes β₁-AR-Gi coupling after 5 minutes of stimulation [16], an ISO stimulation only exhibits obvious β₁-AR-Gi binding in 15 minutes [12]. This phenomenon still requires in-depth research. (2) Would Gi couple β₁-AR under physiological conditions? We speculate that NE can also induce β₁-AR binding to Gi under physiological conditions, because it has been shown that NE can induce β₁-AR binding to Gi in CHO cells [21]. However, there must be extremely stringent conditions for this, and the exact conditions required need to be further investigated. In the above-mentioned studies, all the combinations of β₁-AR and Gi were formed under certain experimental conditions and disease models. Yet, does β₁-AR also bind with Gi when catecholamines activate β₁-AR under normal physiological conditions? That is also a question which needs to be answered in the future.
The Selection of β1-AR on Gi

The switching of G protein refers to the ability of certain GPCRs to bind multiple G proteins to convert from one type of G protein to another when stimulated by a ligand. β2-AR is a typical representative of such kind. When β2-AR is activated by ISO, the receptor interacts first with the Gs protein and then transmits the signal through the AC-cAMP-PKA pathway. PKA, as a protein kinase, phosphorylates β2-AR, which in turn promotes the switching of β2-AR from Gs protein to Gi [23]. G protein switching following β2-AR activation was demonstrated by an experimental method of neonatal rat cardiac myocytes contraction rate, which occurred approximately 15 minutes after ISO stimulation [11, 12].

However, the mechanism of β1-AR biased binding to Gi deviates from that of β2-AR. In HEK293 cells, although stimulation by ISO causes β1-AR to bind with Gi, it is not sufficient to show that β1-AR can bind with Gs and Gi sequentially like β2-AR [12]. In addition, under the action of carvedilol, the Gs/Gi switch of β1-AR differs from that of β2-AR in HEK293 cell [16]. The principal factors of β2-AR G-protein switching, the Gs-receptor binding and the receptor phosphorylation by PKA, have little effect on the G protein switching regarding β1-AR. Rather, the C-terminal of β1-AR plays a key role in determining the binding of Gi protein to β1-AR. Through interchanging the C-terminal of β1-AR and β2-AR, two chimeras were obtained: β1/2-AR (β1-AR chimeric β2-AR C-terminal) and β2/1-AR (β2-AR chimeric β1-AR C-terminal). Carvedilol promotes the recruitment of Gi to β1-AR, but not β2/1-AR or β1/2-AR [16]. Previous studies also showed that the PDZ domain at the C-terminal of β1-AR hinders the binding of Gi to β1-AR in neonatal myocytes [12]. These results suggest that while the β1-AR C-terminal influences the selection of Gi by the β1-AR, the act is not sufficient by C-terminal alone. However, the results of the Lefkowitz’s group [21] showed that PKA is involved in the Gs/Gi switching of β1-AR in CHO cells. It was found that in CHO cells, NE activation of β1-AR increased ERK activity. Both the PKA inhibitor H-89 and the Gi inhibitor PTX reversed this phenomenon. The results are different between different groups concerning the question of whether PKA affects the switching of Gs/Gi in β1-AR. We speculate that the difference in experimental conditions between the groups may have led to the difference in conclusions. In CHO
cells, NE activation of $\beta_1$-AR increases ERK activity. Both PKA blocker H89 and Gi blocker PTX reversed this phenomenon [21]. In HEK293 cells, carvedilol biasedly induced G$i$ to bind to $\beta_1$-AR. Mutating the PKA phosphorylation site of $\beta_1$-AR or using H-89 did not affect the binding of G$i$ to $\beta_1$-AR (Table 1) [16]. This indicates that the effect of PKA on Gs/Gi switching of $\beta_1$-AR is different under different experimental conditions. But one thing for sure is that G protein is also switched after $\beta_1$-AR activation.

However, the above studies were carried out in cells, and the ligand appertained to $\beta_1$-AR is relatively uniform (only by ISO or carvedilol stimulation). In animals, however, besides the exogenous administration of ISO or carvedilol, the local catecholamines in the body are also $\beta_1$-AR agonists. The results of carvedilol-treated mice with heart dysfunction have proved that carvedilol biasedly activates $\beta_1$-AR-Gi and exerts cardioprotective effects through the cGMP-PKG pathway [17]. It is noteworthy that catecholamines, as orthotopic ligands, activate $\beta_1$-AR to trigger its biased binding to Gs; carvedilol, also as an orthotopic ligand [24], rather promotes the preferential binding of $\beta_1$-AR to Gi. Therefore, it raises a question: how does the switching of $\beta_1$-AR-G protein engage in vivo? It is reasonable to speculate that Gs is coupled to $\beta_1$-AR in the body in response to stimulation with catecholamines, yet carvedilol switches $\beta_1$-AR-bound G protein from Gs to Gi. In this process, the C-terminal of $\beta_1$-AR is involved in the selection of G protein. However, the specific mechanism of $\beta_1$-AR G protein switching remains to be further explored.

**The Conformation of $\beta_1$-AR-Gi Complex**

Recently, Huang’s group [10] determined the conformation of turkey $\beta_1$-AR-Gi complex at the resolution of 3.0 Å with the full agonist isoproterenol. The structural analysis of this complex has greatly helped us to understand the binding mode of $\beta_1$-AR to Gi.

In this complex, $\beta_1$-AR adopts the same active-state conformation as the $\beta_1$-AR-Gs complex [10, 25]. Briefly, transmembrane 6 (TM6) is rotated outward by ~14 Å, which is the utmost structural change in the cytoplasmic side of $\beta_1$-AR, while TM7 is moved inward by ~5 Å [25]. In addition, the conserved D(E)RY motif on TM3 and the conserved NPxxY motif on TM7 both undergo
conformational changes as well, in order to couple Gi in the transducer binding pocket formed by TM3, TM5, TM6 and intracellular loop 2 (ICL2). The function of transducer binding pocket is to hold the C-terminal α5-helix of the Ras-like domain on Gα subunit. Gα, containing a Ras-like GTPase domain and an α-helical domain, is one of the three subunits of G protein. These two domains regulate the release of GDP from Gα and the binding of GTP \[8, 26\]. In the β1-AR-Gi complex, the α-helical domain is rotated open by ~79°, and thus is displaced ~37 Å of its mass center relative to the Ras-like GTPase domain: this is the conformational change that is of the most significance in the Gαi subunit [10]. Similarly, the conformational change of the α-helical domain also exists in a β1-AR-Gs complex. However, in Gs, the α-helical domain has a rotation of ~96° instead, and the distance between mass centers is ~38 Å [25].

Although β1-AR can bind with both Gs and Gi, the selectivity of β1-AR to Gs is significantly higher than that of Gi under ISO stimulation [10, 27]. Multiple distinct regions of β1-AR, like ICL2 and ICL4 (TM7/TM8 linker in C terminal), contribute to the determinants of G-protein biased selection. As a result, based on the analysis of complex conformation, the overall structure of β1-AR-G protein complex determines the selectivity of G protein [10].

In summary, on one hand, β1-AR is in an activated state under ISO stimulation, exposing the transducer binding pocket to couple Gs or Gi protein; On the other hand, carvedilol, a special beta-blocker with biased agonist activity [24], can bias the β1-AR to bind with Gi [16, 17, 24, 28]. The phenomenon is a stirring of curiosity to us: what is the conformation of the carvedilol-β1-AR-Gi complex? This perplexity can only be explained by analyzing the structure of the carvedilol-β1-AR-Gi complex. Gi coupling requires the outward movement of TM6 of β1-AR, which is a hallmark of GPCR activation [10]. Therefore, carvedilol-bound β1-AR couples to Gi, suggesting that carvedilol has the ability to induce β1-AR to an active conformation. Unfortunately, the structure of β1-AR-Gi complex with carvedilol as a ligand has not yet been resolved. Recently, human β1-AR was analysed [29], which provided a better structural basis for the development of drugs that promote biased agonism of β1-AR-Gi.

**Two β1-AR-Gi Signaling Pathways**
\( \beta_1 \)-AR-Gi-EGFR-ERK pathway

In 2008, Rockman’s group [22] provided evidence proving that carvedilol can transactivate epidermal growth factor receptor (EGFR) and its downstream signaling protein extracellular signal-regulated kinase (ERK) after biased \( \beta_1 \)-AR-\( \beta \)-arrestin activation. Ten years later, they [16, 22] further developed this particular signaling pathway: carvedilol activates \( \beta_1 \)-AR to bind Gi, and then recruits \( \beta \)-arrestin and Src to transactivate the EGFR-ERK pathway (Figure 1). Additionally, \( \beta_1 \)-AR has been shown to activate the EGFR-ERK signaling pathway by \( \beta \)-arrestin transactivation in response to chronic catecholamines stress, thereby producing cardioprotective effects [30, 31]. These facts offer sufficient theoretical foundation for the future usage of this signal pathway to treat heart diseases.

\( \beta_1 \)-AR-Gi-PI3K-Akt-NOS3-cGMP-PKG pathway

Recent research has revealed a new signaling pathway through animal and cell experiments: \( \beta_1 \)-AR-Gi-PI3K-Akt-nitric oxide synthase 3 (NOS3)-cGMP-PKG (Figure 1) [17]. This study showed that carvedilol increased the contractile shortening in adult mice left ventricular myocytes, although this increase was only 45% of that caused by ISO. What is fascinating is that the carvedilol-induced contraction cannot be blocked by the PKA inhibitor PKI, but by the PKG blocker DT-2. Two pathways, cAMP-PKA and cGMP-PKG, are detected by fluorescence resonance energy transfer during carvedilol stimulation, showing that carvedilol significantly enhances the cGMP-PKG signal but has little effect on the cAMP-PKA signal. Moreover, the carvedilol-induced cGMP-PKG signal is blocked by Gi inhibitor PTX, PI3K inhibitor LY294002 and Akt inhibitor MK2206.

Nitric oxide synthase (NOS) is the key enzyme in nitric oxide (NO) production. NO is a promotor which triggers cGMP synthesis by activating soluble guanylyl cyclases (sGCs) [32, 33]. The NOS that function in the heart are mainly NOS1 and NOS3 [34]. By interfering the expressions of NOS1 and NOS3 in adult left ventricular myocytes, the researchers revealed that carvedilol initiates cGMP signaling through the NOS3 pathway. Carvedilol, through the biased activation of the aforementioned signaling pathway, plays a cardioprotective role. Additionally, carvedilol significantly improves cardiac
functions, such as increased ejection fraction, improved fraction shortening, and improved systolic shortening, in high-fat diet-induced cardiac insufficiency mice.

Although the two signal pathways are different, their function is to protect the heart. It may help clarify the mechanism of carvedilol in the treatment of heart diseases.

Conclusions and perspectives
As a GPCR, the key to the function of β₁-AR lies in its binding with G protein to achieve the purpose of information transmitting [35]. In 1985, β₁-AR was shown to bind to Gs [36]. The majority of the subsequent studies focused on the β₁-AR-Gs pathway, for example, explaining the mechanism of β₁-AR function [14] and verifying it as a therapeutic target for heart disease [37, 38]. After preliminary studies in 2002 which made it clear that β₁-AR can actually recruit Gi, growing evidence supported that β₁-AR can indeed bind to Gi and play a cardioprotective role through different signaling pathways [10, 16, 17]. The whole set of discoveries prompted us to re-evaluate the preference of β₁-AR over G protein. Therefore, we used “G protein coupled receptor” as the word base and modified it appropriately to emphasize Gi-protein-coupled β₁-adrenergic receptor.

Of course, there are many factors which influence the specificity selection process of G protein, such as the conformational changes of β₁-AR [10, 25], the choice of ligands (NE, ISO, carvedilol) [10, 12, 17], and PKA [16, 21]. Both the β₁-AR agonists NE and ISO, and blocker carvedilol can induce β₁-AR binding to Gi [10, 12, 16, 17, 21]. In addition, the β₁-AR conformation must be suitably altered to expose the transducer binding pocket to bind with Gi proteins [10]. As for the role of PKA, the findings are contradictory in different studies. In CHO cells, PKA affects the selection of Gi by the receptor when NE activates β₁-AR [21]. However, in HEK293 cells, the effect of PKA is trivial when carvedilol is biased to activate β₁-AR-Gi [16]. This further suggests that the binding of β₁-AR to Gi requires strict conditional constraints. Thus, it is a coordinated action of multiple factors that drives β₁-AR to finally bind to Gi protein.
In the meantime, there are still many issues to be resolved in the whole process of re-evaluating the β₁-AR-Gi coupling, including 1) the prerequisites for β₁-AR-Gi binding; 2) the β₁-AR-Gs/Gi switch mechanism; 3) analysis of carvedilol-β₁-AR-Gi complex.

The discovery of the β₁-AR-Gi pathway is exciting. The biased binding of β₁-AR to Gi and its cardioprotective effects provide vast vistas for the treatment of cardiac conditions. Currently, the cardioprotective effect of the β₁-AR-Gi pathway is mainly achieved by carvedilol. Clinical studies have shown that, as a beta-blocker, carvedilol is also widely used in the treatment of hypertension [39, 40], cirrhosis and gastroesophageal reflux [41]. Basic studies have shown that carvedilol inhibits the progression of hepatic fibrosis in the digestive system in mice [42]. In the nervous system, carvedilol also has neuroprotective effects in diabetic neuropathy [43]. Although there is a lack of direct evidence whether carvedilol plays a part via the β₁-AR-Gi pathway in the above diseases, the very existence of this pathway provides a theoretical basis for exploring strategies to treat these diseases. Hopefully, the treatment targeting β₁-AR will not only block the Gs pathway, but also provide the full value of β₁-AR-Gi pathway in bringing welfare to patients with heart diseases.

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Conflicts of Interest
The authors declare that they have no conflict of interest.

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**Figure Legend**

**Figure 1. Different signaling pathways, same cardioprotective effect**

EGFR-ERK pathway and NOS3-cGMP-PKG signaling can be activated by biased activation of β1-AR-Gi pathway. Both signaling pathways play a role in cardiac protection.
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<td>CHO</td>
<td>NE</td>
<td>EKR phosphorylation</td>
<td>Both PKA inhibitor H-89 and Gi inhibitor PTX can reverse EKR phosphorylation caused by NE.</td>
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<td>PKA does not affect Gs/Gi switching of β&lt;sub&gt;1&lt;/sub&gt;-AR</td>
<td>HEK293</td>
<td>carvedilol</td>
<td>Binding of Gαi to β&lt;sub&gt;1&lt;/sub&gt;-AR</td>
<td>Neither mutating the PKA phosphorylation site of β&lt;sub&gt;1&lt;/sub&gt;-AR nor using H-89 affects the binding of Gi to β&lt;sub&gt;1&lt;/sub&gt;-AR.</td>
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Highlights

β₁-adrenergic receptor (β₁-AR), a member in the family of G-protein-coupled receptors, is a transmembrane receptor of great significance in the heart.

Preceding views. It is believed that β₁-AR couples to Gs protein and transmits the signal through second messenger cAMP. However, increasing evidence shows that β₁-AR can also bind with Gi protein in addition to Gs. This review summarizes the discovery of β₁-AR-Gi pathway, including the evidence that supports β₁-AR's capability to couple Gi, details of the transduction process and functions of β₁-AR-Gi signaling pathway.

- Previously, β₁-AR was thought to bind only with Gs, but an increasing number of studies have shown that β₁-AR can also bind with Gi.
- Both in vivo and in vitro experiments have demonstrated that β₁-AR can bind with Gi and that carvedilol can bias the binding of both.
- The biased activation of β₁-AR/Gi pathway by carvedilol may exert cardioprotective effects via EGFR-ERK pathway and NOS3-cGMP-PKG signaling.