European Journal of Pharmacology 794 (2017) 234–245

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Salicylic acid retention impairs aspirin reactivity in type 2 diabetes

Haowen Zhang[a,b,1], Hao Xie[c,1], Xiao Zheng[c,d], Yingying Chai[c], Zhiyuan Tang[c], Hanyu Chen[a,b], Feiyan Li[c], Heier Christoph[e], Jiandong Chen[b], Weixin Sun[a,b], Hui Ye[c], Shiguang Wang[a,b], Haiping Hao[c,*], Xiaohu Chen[b,*]

[a] First Clinical Medical College, Nanjing University of Chinese Medicine, Nanjing, China
[b] Department of Cardiology, Jiangsu Province Hospital of Traditional Chinese Medicine, the Affiliated Hospital of Nanjing University of Chinese Medicine, 155 Hanzhong Road, QinHuai District, Nanjing 210029, China
[c] State Key Laboratory of Natural Medicines, Key Lab of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, 24 Tongjiaxiang, Gulou District, Nanjing 210009, China
[d] Department of Pharmacy, Jiangsu Province Hospital of Traditional Chinese Medicine, the Affiliated Hospital of Nanjing University of Chinese Medicine Nanjing, China
[e] Institute of Molecular Biosciences, University of Graz, Graz, Austria

ARTICLE INFO

Keywords:
HAPR
Diabetes
Salicylic acid
Cyclooxygenase-2
Platelet

ABSTRACT

High on-aspirin platelet reactivity (HAPR) has been associated with compromised aspirin efficacy in patients with diabetes suffering from acute cardiovascular events, but the key mechanisms remain elusive. The objective of this study was to uncover the potential link between pathogenic accumulation of salicylic acid (SA), the major metabolite of aspirin, and HAPR in diabetic state. Aspirin failed to inhibit platelet CD62P expression and thromboxane (TX) B_2/6-keto-prostaglandin (PG) $F_{1\alpha}$ ratio in a type 2 diabetes mellitus (T2DM) mice model, particularly in the female, which were unanimously accompanied by significantly higher plasma SA concentrations. Pre-administration with SA increased both platelet CD62P expression and TXB_2/6-keto-$PGF_{1\alpha}$ ratio in female T2DM mice, while pretreatment with $NaHCO_3$ caused the opposite effect. On the *in vitro* human umbilical vein endothelial cells (HUVECs)-platelet interaction assay, SA suppressed inflammation-induced cyclooxygenase-2 upregulation on HUVECs and attenuated their inhibitory effect on platelet aggregation in a dose-dependent manner. The prolonged retention of SA in diabetes may be partially explained by the downregulation of various SA efflux transporters in the kidney and the decreased urine pH. Importantly, in female aspirin non-responsive patients, the trough plasma concentration of SA are markedly increased with T2DM treated with long-term aspirin, and TXB_2/6-keto-$PGF_{1\alpha}$ ratio and uric acid level in plasma are positively correlated with SA concentration. Our findings support that the accumulation of SA represents an important factor in causing HAPR in diabetes, and that targeting impaired SA excretion may become a novel intervention strategy to diabetes-associated HAPR.

1. Introduction

Aspirin is the cornerstone of anti-platelet therapy in the secondary prevention for stroke, myocardial infarction and other cardiovascular events. However, a large proportion, up to 40%, of patients taking aspirin may still suffer from cardiovascular events. Such patients are considered as aspirin resistant (AR) or high on-aspirin platelet reactivity (HAPR) (Breet et al., 2010; Hankey and Eikelboom, 2006). Previous studies suggested that a long list of causal factors, like poor compliance, co-existing diseases such as diabetes, gender and drug-drug interactions, are related to impaired outcome of aspirin treatment (Floyd and Ferro, 2014). However, deep insights into this multiplexed clinical issue are largely lacking.

Large scale clinical trials have confirmed diabetes as an independent high risk factor of HAPR (Ertugrul et al., 2010; Fateh-Moghadam et al., 2005; Simpson et al., 2014; Yassine et al., 2010), although the underlying causes and mechanisms remain largely unclear and controversial. It is well known that platelet activation depends on the ratio of thromboxane (TX) A_2, mainly derived from the platelet cyclooxygenase (COX)-1, to prostaglandin (PG) I_2 mainly produced by endothelial COX-2, and indeed, TXA_2/PGI_2 has been used as a clinical surrogate of aspirin response (Sakata et al., 2013). Therefore, it is

* Corresponding authors.
E-mail addresses: hhp_770505@hotmail.com (H. Hao), chenxhdoctor@126.com (X. Chen).
[1] contributed equally to this work.

http://dx.doi.org/10.1016/j.ejphar.2016.11.042
Received 19 September 2016; Received in revised form 22 November 2016; Accepted 24 November 2016
Available online 25 November 2016

reasonable to propose that the dynamic balance between platelet COX-1 and endothelial COX-2 may represent a pivotal factor in determining aspirin response in various pathological conditions including diabetes. In diabetes, both the platelet COX-1 activity and endothelial COX-2 activity are induced; the latter may represent an adaptive mechanism for combating the platelet over-activation (Szerafin et al., 2006). We thus hypothesized that aspirin responsiveness in diabetes might depend on the balance of platelet COX-1 and endothelial COX-2 and that factors of potential COX-1 inhibitory effect may explain HAPR in diabetes.

After oral administration, aspirin undergoes a quick metabolism by aspirin esterase in the gastrointestinal tract, blood and liver to salicylic acid (SA), which represents the major metabolite of aspirin in circulation (Hutt et al., 1986; Patel et al., 1990). Actually, SA is manifested as the predominant form of aspirin reaching systemic circulation systems, because aspirin is almost completely de-acetylated to SA with a half-life of only 15–20 min (Miners, 1989). Aspirin is largely excreted out in the form of SA via renal transport, mediated by renal organic anion transporters (OATs), sodium-coupled monocarboxylate transporters (SMCTs) and urate transporter 1 (URAT1) (Dresser et al., 2001; Ganapathy et al., 2008; Hosoyamada et al., 2004). Of particular interest, it was previously found that SA, at a high concentration, could inhibit the inflammation induced endothelial expression of COX-2 (Xu et al., 1999). We thus asked whether increased plasma concentration of SA may underlie HAPR in diabetes. Because SA is mainly excreted via the kidney transporters and it is well known that the kidney is one of the main organs influenced by diabetes, the abnormal accumulation of SA is highly possible in the context of diabetes.

Here we found that SA accumulation was closely associated with HAPR in both a female type 2 diabetes mellitus (T2DM) mice model and female patients. Mechanistically, we show that the accumulation of SA, at a pathologically relevant concentration, is sufficient to inhibit adaptive COX-2 up-regulation in aorta abdominalis and interleukin1β (IL-1β) stimulated human umbilical vein endothelial cells (HUVECs), thereby decreasing the level of PGI_2 and exacerbating HAPR in diabetes.

2. Materials and methods

2.1. Mice and induction of T2DM model

Specific pathogen free (SPF) C57BL/6 J mice (male and female, 8 weeks old) were obtained from Translation Medicine Center of Yangzhou University (Yangzhou, China). The animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University and have been carried out in accordance with the NIH guidelines. Animals were housed in an air-conditioned room (25 °C) under a 12-h light/dark cycle for 1 week before experiments and allowed water and standard chow *ad libitum*. Mice were randomly divided into different groups according to body weights in all the experiments.

For the induction of type 2 diabetes model in mice, a group of mice received high-fat diet (HFD) feeding (19.7 kJ/g, 45% of energy as fat, Synergistic Pharmaceutical Biotechnology Companies, Jiangsu, China) for 3 weeks. After a 16-h fasting, weight matched mice were further challenged by a single intraperitoneal injection of streptozotocin (STZ, 100 mg/kg) (Sigma, St. Louis, MO, USA) dissolved in 0.1 M cold citrate buffer (pH 4.5). Five weeks after STZ administration, mice displayed hyperglycemia, hyperlipidemia and insulin resistance were selected and defined as T2DM mice for the experiment, as previously described (Mu et al., 2006).

2.2. Treatment protocols

In the pharmacological investigations and the detection of valley SA concentration at 24 h, normal mice (male, n=14; female, n=14) were randomly divided into two groups and treated with 0.5% CMC-Na or aspirin (13 mg/kg, i.g, scaled from the clinic dose 100 mg/kg/day applied for patients with T2DM) (Lemkes et al., 2012) dissolved in 0.5% CMC-Na for 10 days, respectively; T2DM mice were divided into four groups and treated with vehicle (0.5% CMC-Na) for 10 days (male, n=7; female, n=7), aspirin (13 mg/kg, i.g) (dissolved in 0.5% CMC-Na) for 10 days (male, n=7; female, n=7), aspirin (13 mg/kg, i.g) for 1 d followed by 9 days of SA (10 mg/kg, i.g, equal molar concentration of aspirin) (female, n=7), or $NaHCO_3$ (600 mg/kg, i.g) (Ren et al., 2014) plus aspirin (13 mg/kg, i.g) for 10 days (female, n=7), respectively. All mice were killed at indicated time points after the last administration. Blood collected used for the pharmacological study and the 24 h valley salicylic acid concentration detection and the kidney and aorta abdominalis were excised and placed in PBS or snap frozen in liquid nitrogen and stored in −80 °C until further processing.

2.3. Pharmacokinetic study of SA

To study SA pharmacokinetics in T2DM mice, blood from the canthus venous was collected in 1.5 ml centrifuge tube with heparin at 0.25, 0.5, 1, 2.5, 5, 8, 12 h in normal and T2DM mice (male, n=56; female, n=56; 4 mice *7 time points for each experimental group) on the last day after aspirin (13 mg/kg, i.g) administration for 10 days. For the determination of SA concentration, plasma samples were precipitated with acetonitrile containing 0.1% formic acid and the internal standard (IS, 3-methyl-salicylic acid, 100 ng/ml). The turbidity systems was vibrated, centrifuged and recentrifuged before being analyzed by liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) by using a Shimadzu high-performance liquid chromatography apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole mass spectrometry via an electrospray ionization interface. The chromatographic separation was achieved on an Agilent C_{18} column (150×2.1 mm, 3.5 μm) (Agilent Technologies, USA), protected by a SecurityGuard (Phenomenex Inc. CA. USA). The mobile phase comprising 0.1% formic-acid solution (A) and acetonitrile (B) was delivered at a flow rate of 0.2 ml/min using a gradient program as follows: initial 15% B for 2 min, linear gradient 10–90% B from 3 to 10 min and to 90% B until 15 min, and then returned back to initial 15% in 2 min and maintained for a further 2 min for column balance. Selective ion monitoring was performed in the negative mode for the determination of pseudomolecular ions of SA at m/z 136.95 and the IS at m/z 150.95. The amounts of SA in plasma are expressed as micrograms per milliliter.

2.4. Biochemical analysis

Serum was collected from the canthus venous after centrifugation at 2000*g* for 5 min. Glucose concentrations were detected rapidly in blood taken from the tail tip vein of a 12-h fasting mice using glucose meter (USA Optium Xceed), while total cholesterol (TC) and triglycerides (TG) and were determined by assay Kit according to the manufacturer's instructions (Jiancheng, Nanjing China), Insulin level was determined by using a radioimmunoassay kit (The northern Biotechnology Co., Beijing, China), respectively. TXB_2 and 6-keto-$PGF_{1\alpha}$ contents were determined by radioimmunoassay and the kits were supplied by Institute of Radioimmunoassay, Nanjing Clinical Nuclear Medicine Center. Urine was collected into tubes with sodium azide over a 24 h period in metabolic cages. The urinary pH of mouse was detected using pH meter (sartorius, Beijing). The concentrations of inflammatory cytokines (IL-6, IL-1β) in serum of T2DM mice were determined by ELISA (ExCell Biology, Shanghai, China).

2.5. Flow cytometry analysis of platelet

Platelet-rich plasma was separated from 3.5% acid citric acid-