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Review Article

Platelet-Activating Factor (PAF) and Trimethylamine Oxide (TMAO) - Silent Provocateurs of Genuine Coronary Spasm

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Abstract

The so-called genuine coronary artery spasm leading to myocardial infarction is one of the most insidious diseases that threaten human life, usually overtaking a person in the prime of life and, it would seem, without any apparent reason. In fact, such a reason is always there. A relatively short but powerful contraction of the coronary artery leads to a sharp restriction or complete cessation of coronary blood flow, fatal heart rhythm disturbance and cardiac arrest. The question is what provokes a sudden contraction of the smooth muscles of the vessel and the cessation of blood flow to a significant part of a vital organ. What previous changes in the body and directly in the myocardium can lead to such a development of events - tissue hypoxia, endothelial dysfunction, blockade of glycolysis and outgoing potassium current, increased activity of C and Rho-kinases? Or are there other, as yet little-known, culprits of fatal coronary spasm? Hypoxia is indeed a potent factor that leads to the formation and release of platelet-activating factor (PAF), and PAF acts as a trigger for coronary vasospasm in pig coronary arteries. This compound binds to specific receptors and promotes the activation of phospholipase C and the subsequent formation of inositol 1,4,5-triphosphate and diacylglycerol. The subsequent intracellular calcium release and activation of protein kinase C cause a pronounced contraction of the arteries, which is preserved in a Ca^{2+}-free solution, but is completely eliminated by BN 52021, a potent PAF receptor blocker. Another possible candidate for the role of coronary spasm initiator is our intestine, or more precisely its microbiota. Throughout human life, the intestinal microbiota continuously synthesizes and secretes trimethylamine (TMA) and trimethylamine-N-oxide (TMAO), the chemical structure of which is remarkably similar to that of tetraethylammonium, a known potassium channel blocker. The cumulative inhibitory effect of TMAO ions on the outward potassium current in vascular smooth muscle cells can lead to depolarization of cell membranes and a decrease in their excitability threshold. This creates ideal conditions for the development of smooth muscle hypercontractility and promotes an abnormally high increase in the level of tonic tension in the vascular wall in response to the appearance of a necessary external stimulus, such as a decrease in the oxygenation level. There is evidence that β-hydroxymethylbutyrate helps reduce the formation of trimethylamine in the intestine and helps normalize the qualitative composition of the microbiota, and also prevents the formation of trimethylamine from its precursors contained in food: choline, betaine, carnitine, lecithin, etc. Due to this, the concentration of trimethylamine in the blood and the possibility of its conversion to TMAO are significantly reduced. Blockade of flavin monooxygenase 3 by diindolylmethane, a natural substance from cruciferous vegetables that ensures the conversion of TMA to TMA-O, can significantly enhance the positive effect of hydroxymethylbutyrate, as well as additional administration of leucine, which competes with carnitine. Therefore, a more pronounced effect should be inherent in a combination containing hydroxymethylbutyrate, diindolylmethane and leucine.

Keywords: *Coronary Artery Spasm (CAS); Trimethylamine (TMA); Trimethylamine-N-Oxide (TMAO); β-Hydroxymethylbutyrate; Platelet-Activating Factor (PAF)*

Introduction to the Problem

Genuine coronary artery spasm (CAS) as it leads to myocardial infarction, angina and cardiac arrhythmia is one of the most insidious diseases that threaten a person's life overtaking him, as a rule, in the prime of his life and, it would seem, for no apparent reason. In addition, even a post-mortem autopsy is far from always able to give a clear and final answer to the cause of death of a person. However, such a reason always exists. For example, such a chain of events - a short-term but powerful contraction of a section of the epicardial coronary artery, leading to a sharp restriction or cessation of coronary blood flow, the formation of an ischemic zone with other electrophysiological properties, a fatal disturbance of the heart rhythm and cardiac arrest. Sudden death. The question is what served as a trigger that caused a large vessel to suddenly contract and stop blood flow to a significant part of a vital organ. What previous changes in the body and myocardium can lead to such a development of events? Can tissue hypoxia lead to fatal contraction of the coronary artery? What is the role of the endothelium malfunction, perivascular tissues and inhibition of glycolysis and/or outward potassium currents in the development of unwanted coronary artery constriction? How do C and Rho kinases influence the development of vascular spastic conditions? Is it possible to explain the development of CAS in a purely mechanistic way, i.e. in terms of hydromechanics? Alternatively, are there some other, still little known culprits of the fatal spasm of the coronary vessels? Finally, the main thing. What are the prospects for the prevention and development of coronary spasm? We tried to provide answers to some of these questions, while understanding the complexity of the task set before us.

The existing data allow us to imagine the possible scenario for the development of genuine coronary spasm as follows. One of the most common causes is an apparently severe imbalance between myocardial oxygen demand and consumption (oxygen deficiency). With a moderate decrease the required degree of oxygenation of smooth muscles, coronary arteries dilate, with a strong decrease they contract. The threshold for the development of hypoxic spasm can vary greatly from person to person. All other factors, including endothelial dysfunction, inhibition of glycolysis, diminished outward currents, activation of C and Rho kinases, either play the role of sensitizers to hypoxia or contribute to an increase in calcium sensitivity of smooth muscle myofilaments related hypercontractility or other not yet known oxygen-dependent mechanisms of coronary spasm development. Based on this, immediate re-oxygenation could be the first aid, but technically, in most cases, this is difficult to provide. Treatment or coronary spasm prevention should be aimed at eliminating the hypercontractility sensitizers or correction the mechanisms mentioned above.

Can we finally give an intelligible answer to the question posed at the beginning of this article - why a blood vessel with no visible signs of pathology suddenly "commits an act of suicide" in a state of rest and in the absence of any stress factors. The only thing we know so far for sure about the nature of coronary spasm is that it is, at its core, a complex multifactorial process.

For many years, there has been a point of view among oncologists that the appearance of an oncological disease in a person is the result of the summation of mistakes made by this particular person during his entire life preceding this fatal event. Many oncologists believe that our body prepares cancer for most of our lives. This disease rises in connection with frequent malnutrition, hormonal dysfunctions, various excesses, intoxications, infections and parasitic infestations, as well as repeated injuries that are subsequently detected during x-ray examination or autopsy.

It seems to us that a similar situation is observed in the case of coronary spasm. It has now become clear that large coronary arteries respond to a decrease in oxygenation by contraction. Healthy smooth muscle cells of human epicardial coronary arteries have a pronounced spontaneous activity, which in itself already indicates a certain degree of readiness for the development of spastic contraction, and are able to reduce their lumen under hypoxic conditions. This is already a fact. This process can happen many times in a person's life. because it is a normal physiological reaction. No one is surprised by the fact that a similar phenomenon occurs during hypoxia in the vessels of

the lungs. Hypoxic contraction of large coronary arteries increases the pressure gradient in the coronary bed and, subject to mandatory relaxation of resistive vessels, will lead to an increase in coronary blood flow and compensate for the lack of oxygen. If the smooth muscles of the arterioles are compromised in one way or another (inhibition of glycolysis and/or potassium outward currents, high activity of C - and Rho kinases, etc.), such vasodilation will not occur, and then the contraction of large arteries will increase, leading to a fatal decrease in myocardial cell perfusion.

Our main task was to try to identify the main, possibly universal, trigger mechanism initiating the development of coronary spasm. It is quite possible that this is exactly the same mechanism as in the case of the tumor process. Like a bomb with a smoldering fuse, it is formed in the pre-infarction period of a person's life, not manifesting itself until the intensity of some external stimulus exceeds the regulatory and compensatory capabilities of the myocardial blood supply system. Since this pathological mechanism does not manifest itself, then most likely it may be some factor inherent in our body, the presence of which is inalienable in our life.

Thus, we need to figure out what is the main mechanism underlies the formation of coronary spasm. It cannot arise just like that, out of nothing. Some biochemical or electrophysiological processes, occurring at the molecular level, must necessarily be identified, which should serve as the basis for a sudden hypercontraction of the coronary artery.

Furthermore, there is compelling evidence that mediators produced in the hypoxic (ischemic) and post-hypoxic (reoxygenated) region not only exacerbate ischemia-induced contractile depression (and possibly arterial dysfunction), but can also diffuse into the undamaged, "healthy" part, thereby causing global problems with the myocardium at the cellular level [1].

Platelet-activating factor (PAF) as a possible trigger of coronary spasm development

In this regard, it is appropriate to recall our joint studies with Pierre Braquet (France) on the role of the so-called platelet-activating factor (PAF) in the development of hypoxic coronary spasm. Everyone knows that phosphatidylcholine is one of the main structural and functional phospholipids in the human body. In fact, it is the basis of our life. Even more surprising is the fact that after a series of relatively simple metabolic transformations, they easily turn into PAF, a mediator of many life-threatening pathological processes.

In fact, this compound was first described as a soluble component released from rabbit basophils sensitized with immunoglobulin E, which caused platelet aggregation. However, this phospholipid has an extremely wide spectrum of biological effects. For instance, in various cell systems PAF induces the production of superoxide anion which may inactivate endothelium-derived relaxing factor [2]. Now it is also known as a potent mediator of inflammation, allergic responses, and shock. It has a role as a beta-adrenergic antagonist, an antihypertensive agent, a bronchoconstrictor agent, a hematologic agent and a vasoactive agent.

PAF - a phospholipase A_2-sensitive phospholipid identified as 1-alkyl-2(R)-acetyl-glycero-3-phosphorylcholine. It is established that PLA_2, activation is involved in the biosynthesis of PAF [3]. The activation of PLA_2, is Ca^{2+} dependent, and generally agonists and external influences that stimulate Ca^{2+} mobilization induce the formation and release of PAF. In other words, PAF is an indispensable participant in all processes occurring in a living organism. In the first step, phospholipase A2 (PLA2) acts on phosphatidylcholine producing eicosanoid (arachidonic acid) and lysophosphatidylcholine (LPC). In the second and final step, acetyl residue is transferred to LPC by LPC acetyltransferase to produce PAF (Figure 1).

The source of PAF in human body is an acetylation of ether phospholipids (phosphatidylcholine) after its hydrolysis by PLA, the main sources of PAF in the vascular wall and blood are platelets, polymorphonuclear leukocytes, monocytes, macrophages, eosinophils, mast cells and, of course, endothelial and smooth muscle cells. Contrary to other mediators, is not stored in the cell but is present in the form of the inactive precursor diacylglycerophosphocholine linked to membrane structures.

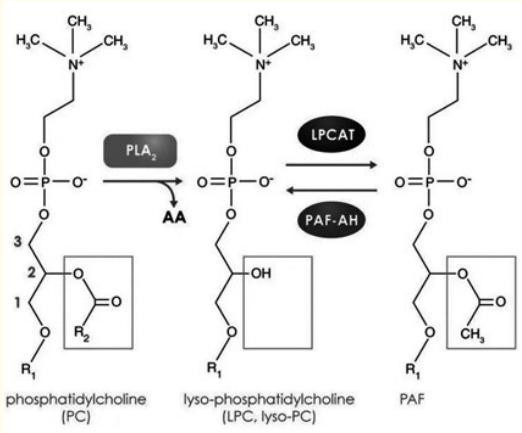

Figure 1: *The biosynthesis of PAF via remodeling of membrane phospholipids (Adapted from Tom Brock [4]).*

There are two pathways to synthesize PAF: remodelling and de-novo synthesis. The remodelling pathway starts with a phospholipid called phosphatidylcholine. It involves substituting an acetyl residue for the long-chain fatty acyl residue a sn-2 of phosphatidylcholine. Another way is formation of platelet-activating factor (PAF, 1-[3H] alkyl-2-acetyl-sn-glycero-3-phosphocholine) from the de novo precursor of PAF, 1-[3H] alkyl-2-acetyl-sn-glycerol).

PAF primary role is to mediate intercellular interactions. A variety of cells synthesizes it, and it binds to the extracellular receptors of other cells, activating them and causing a change in their phenotypes. The PAF receptor is structural characteristics of the rhodopsin (MIM 180380) gene family is a G-protein coupled receptor. There are also intracellular receptors of PAF that are yet to be rigorously characterized. Now it is known also as a potent mediator of inflammation, allergic responses, and shock. It causes a dramatic inflammation of air passage resulting in asthma like symptoms. Production of PAF is inducible by toxins from fragments of destroyed bacteria leading to vasodilation and a drop-in blood pressure resulting in reduced cardiac output and shock.

Platelets, endothelial cells, macrophages, monocytes, and neutrophils continuously produce PAF in low quantity. PAF acetyl hydrolase also known as lipoprotein-associated phospholipase A_2, inactivates the PAF and PAF-like phospholipids, controlling their actions. Its activity increases when specific stimuli activate inflammatory cells. Phospholipase A2 is a biomarker for cardiovascular risk assessment and is associated with unstable atherosclerosis plaques. PAF also correlates with various medical conditions like asthma, stroke, myocardial infarction, certain tumors and cancers, and various other inflammatory conditions [5].

Some important intracellular signalling pathways associated with PAF and related cell responses are shown on figure 2.

In our experiments [6,7] we have studied contractile responses of coronary arteries obtained from humans, pigs and dogs to a decrease in pO_2 of the organ bath solution from 147 to 90 mm Hg (it would seem that this is very moderate hypoxia). Nevertheless, our microelectrode polarography studies showed that with such level in organ bath, the pO_2 in the middle of media drops to 15 mm Hg. As the result, we observed clearly expressed two-phase contractile reaction. Namely, after the phasic contraction, at 7-12 minutes of hypoxia, a slowly developing tonic contraction began to form. Most importantly, human segments with visible signs of atherosclerosis, obtained from

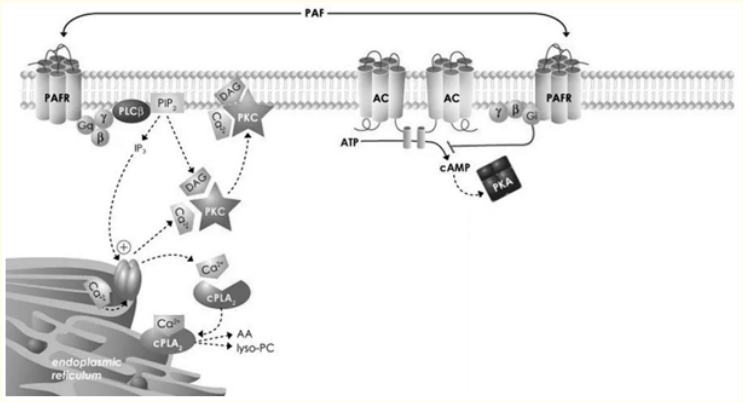

Figure 2: *Intracellular signalling through the PAF receptor (PAFR) The main cellular responses to PAF are leukocyte activation, aggregation of platelets, cell adherence, ROS generation, angiogenesis, expression of COX2, iNOS, IL-6 (Adapted from Tom Brock [4]).*

elderly people, responded to hypoxia with relaxation! By the way, removal of the endothelium led to the abolition of the phase component of the hypoxic contraction of the coronary artery, but did not affect the sustained contraction. The hypoxia-induced contraction of coronary arteries was clearly pronounced in saponin-skinned smooth muscles. What is more, skinned artery contracted under hypoxia even when the concentration of Ca^{2+} in the buffer solution was lower than the minimum necessary to initiate the contraction. This fact clearly indicates to increase in calcium sensitivity of coronary arteries myofilaments under hypoxia. Let's try to answer - why and what does PAF have to do with it.

The suggestion that the contraction of coronary arteries as a result of O_2 deficit is an artefact that only occurs in isolated vascular preparations was quashed when it has been shown [7] that inhalation of hypoxic gas mixture (8% 0_2 - 92% N_2) by anaesthetized dogs leads to a biphasic dilation-constriction response in the coronary vessels. Blood flow increased from 60.2 ± 7.9 to 72.0 ± 10.0 ml/min ($P > 0.05$) followed by a significant reduction, over 10 minutes, to 37.6 ± 10.0 ml/min ($P < 0.01$). It should be noted that with such an oxygen content in the respiratory mixture, pO_2 of the blood would be about 50 - 60 mm Hg.

In above-mentioned papers, we concluded that hypoxia is powerful factor that leads to the formation and release of platelet-activating factor (PAF) in vascular wall. Other investigators have shown in isolated heart experiments a significant release of PAF by cardiac muscle during ischemia-reperfusion [8] and the fact that endothelial cells in culture are capable of producing PAF [9].

PAF acts as a trigger for coronary vasospasm in isolated porcine circumflex epicardial coronary artery. This compound binds with specific receptors and facilitates the activation of phospholipase C (PLC) and subsequent formation of inositol 1,4,5 - trisphosphate (InsP3) and diacylglycerol (DG). The following intracellular calcium release and protein kinase C (PKC) activation caused pronounced artery contraction that appears to be preserved in Ca^{2+} - free solution but completely abolished with BN 52021 (ginkgolide B), a potent PAF-receptor blocker.

Interestingly, treatment with another paf receptor blocker, rupatadine, resulted in worsening of atherosclerosis and undesirable changes in vascular reactivity [10]. This is probably because rupatadine is a multi-target drug (it is also a histamine receptor blocker) and this may be the reason for its negative effects.

Hypoxia is powerful but is not the only factor that leads to the formation and release of PAF in vascular wall. As mentioned above, any factor that stimulates PLA, activity or calcium mobilization in SMC may cause PAF release and trigger coronary spasm. The existence of many such factors and drugs in our lives makes the examination of the role of PAF in coronary vasospasm development very important and, indeed, vital.

In addition, PAF is able can influence directly or through the generation of secondary mediators on cardiac conduction system. This fact may be the basis for the occurrence of sudden arrhythmia. A meta-analysis of preclinical effects of ginkgolides showed that PAF blockers have a significant effect on myocardial protection from hypoxia and the post-hypoxic period [11]. The next figure 3 shows the direct and indirect effects of PAF released during ischemia/perfusion on the contractile function of the heart.

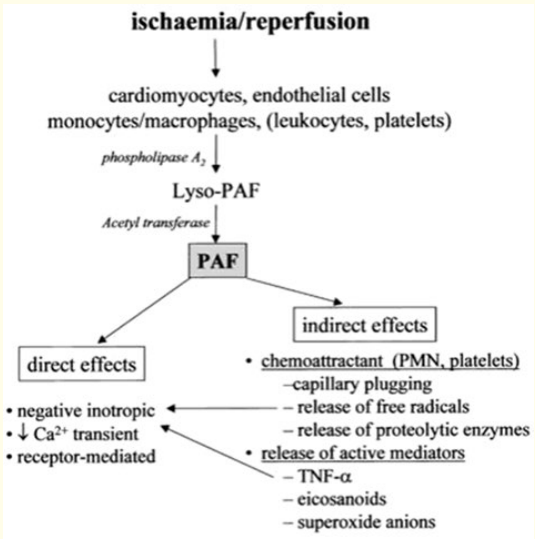

Figure 3: *The role of PAF in contractile dysfunction of the domestic pig heart during ischemia/reperfusion (Adapted from Feuerstein, Boyd, Ezra, Goldstein [12]).*

Thus, we hypothesize that PAF may play a key role in the occurrence (triggering) of sudden coronary spasm.

TMAO (Trimethylamine-n-oxide oxide) and its possible role in coronary spasm development

Another possible candidate for the role of the initiator of coronary spasm comes from our intestines. In the process of human life, the microbiota of its intestines constantly synthesizes the compound trimethylamine (TMA), but largely, another abbreviation is now heard - TMAO (trimethylamine-N-oxide oxide).

TMAO is formed from TMA, which is generated, by the action of gut microbiota from dietary choline and phosphatidylcholine (lecithin) [13] described another pathway that involves the catabolism of L-carnitine through two sequential microbial reactions. Accordingly, L-carnitine is first converted into the intermediate metabolite γ-butyrobetaine and then into TMA, which is subsequently converted into TMAO by host hepatic flavin monooxygenases 3 (FMO3). Red meat, eggs, dairy products and salt-water fish are rich in choline, lecithin, and carnitine and, hence, are a potential source of TMAO. TMA is a gas, which is oxygenated within living animals to form TMAO by flavin monooxygenases (FMO1 and FMO3). TMAO is either then transported to the tissues for accumulation as an osmolyte or, more commonly, cleared by the kidney [14].

TMAO generated by gut microbiome exacerbates impaired glucose tolerance, inhibits hepatic insulin signalling, and promotes adipose tissue inflammation in mice that are maintained on a high-fat high-sugar diet. In animals and humans, TMAO has also been suggested as a strong candidate molecule mediating the development of type 2 diabetes mellitus [15].

Metabolomics allows identification of disease pathogenesis links. Cardiovascular diseases are a leading cause of mortality and morbidity worldwide. Wang, Clipfell, Bennett., *et al*. [16] used a metabolomics approach to generate plasma small molecule metabolism profiles that predict cardiovascular risk. Three phosphatidylcholine metabolites, choline, trimethylamine N-oxide (TMAO), and betaine, were identified as cardiovascular risk markers in a large clinical cohort. Feeding mice with choline, TMAO, or betaine was shown to upregulate multiple scavenger macrophage receptors associated with atherosclerosis, and supplementation with choline or TMAO promoted atherosclerosis. Studies in germ-free mice confirmed the critical role of choline and gut flora in TMAO production, increased cholesterol accumulation by macrophages, and foam cell formation. It was found that suppression of gut flora in atherosclerosis-prone mice inhibited the development of atherosclerosis. The discovery of a link between gut flora-dependent phosphatidylcholine metabolism and the pathogenesis of cardiovascular diseases opens up opportunities for the development of new diagnostic tests and new approaches to the treatment of atherosclerotic heart disease.

In another study, TMAO levels were increased in patients with stable heart failure and elevated levels were associated with increased risk of death [17]. Thus, substantial evidence indicates that elevated TMAO levels are associated with cardiovascular disease.

The data obtained clearly indicate indicated a potential role of the gut-derived metabolite TMAO, which is the product of microbe-dependent conversion of ingested precursors (e.g., choline, betaine and L-carnitine) into trimethylamine (TMA; via TMA lyase). The last one is subsequently absorbed into circulation and converted to TMAO in the liver [16,18,19].

In the first meta-analysis by Shiattarella., *et al*. (2017) it has been shown a strong positive dose-dependent association between TMAO plasma levels and increased cardiovascular risk and mortality [20].

TMAO (as well as PAF) can stimulate superoxide production and cause vascular endothelial dysfunction by uncoupling eNOS [21]. These authors showed that TMAO selectively inhibits EDHF-dependent vasodilation in isolated rat mesenteric arteries without affecting NO-induced vasodilation. These findings may help explain the role of elevated TMAO levels in the development of certain vascular diseases. Taking into account the mechanisms of EDHF-dependent vascular relaxation, it is easy to conclude that TMAO has a direct inhibitory effect on potassium channels. In addition, of course, one cannot ignore the surprising similarity of TMAO with the tetraethylammonium molecule, a well-known blocker of potassium conductivity. This similarity in molecular structure may help to explain the effects of TMA/TMAO based on the structure-activity relationship concept.

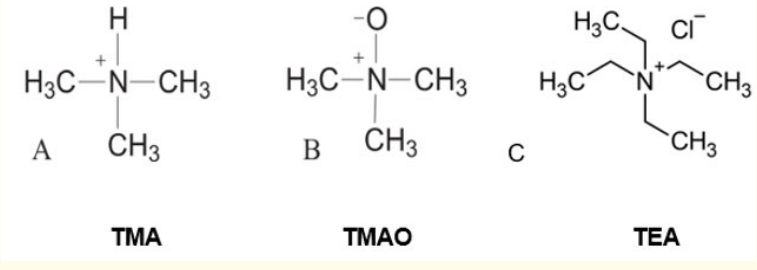

Figure 4: *Chemical structure of TMA (A), TMAO (B) and TEA chloride (C).*

The tetraethyl ammonium (TEA) ion consists of four ethyl groups attached to a central nitrogen atom. The TEA ion is virtually always associated with an anion. It is known that the most commonly used salts of TEA are chlorides, bromides, or iodides, but for convenience, teachers or authors of publications do not always indicate which counter ion they are associated with.

It is well known that TEA application leads to depolarization of smooth muscle cell, contraction and provokes autorhythmic activity. All this taken together creates the necessary conditions for the development of coronary spasm.

Trimethylamine-N-oxide (TMAO) is a small zwitterionic molecule in which the oxygen and nitrogen atoms are charged negatively and positively, respectively. This O-N group can be considered as the hydrophilic part of the molecule. The rest of the TMAO molecule is composed of three methyl groups and can thus be considered as the hydrophobic part. This is the reason for TMAO being called an amphiphilic molecule.

Restini., *et al*. [22] hypothesized that TMA/TMAO may directly alter vascular tone. Perivascular adipose tissue is known to contribute to the regulation of vascular homeostasis and contains flavin monooxygenase 3 (FMO3). The authors used intact and perivascular adipose-free rat thoracic aortas and endothelium-free aorta preparations for contractile measurements. They studied contractile responses to erythromethylamine (TMA)/trimethylamine-N-oxide (TMAO) (1 nM - 0.5 M). Immunohistochemistry was performed to detect and confirm the presence of FMO3 as a product of TMAO. TMA and TMAO induced concentration-dependent arterial contraction. However, at the maximal achievable concentration (0.2 M), TMA-stimulated contraction was of greater amplitude than that induced by TMAO in intact adipose tissue and endothelium. When perivascular adipose tissue was preserved, TMAO-induced contraction was significantly reduced compared with vessels without endothelium, indicating that the endothelium plays a protective role against TMAO-induced contraction. The FMO3 enzyme present in aorta was indeed detected in adipose tissue, but the FMO3 inhibitor, methimazole, did not affect TMA-stimulated contraction in aortas with perivascular adipose tissue. However, the l-type calcium channel blocker nifedipine reduced TMA-induced contraction by approximately 50% compared with controls.

Though a high concentration of these compounds was needed to achieve contraction, the findings that TMA-induced contraction was independent of perivascular adipose tissue and endothelium and mediated by nifedipine-sensitive calcium channels suggest metabolite-induced contraction may be physiologically important. Interesting, that TMA was more potent than TMAO.

Zhang., *et al*. [23] suggest that TMAO causes direct cardiac injury. Gut microbiota dysbiosis has been shown to occur with elevated circulating TMAO levels. In cardiomyocytes, TMAO activates the TGF-b1/Smad3 and p65 NF-kB signaling pathways and reduces energy metabolism and mitochondrial function by affecting the oxidation of pyruvate and fatty acids that participate in the tricarboxylic acid (TCA) cycle. It also negatively affects myocardial contractile function and intracellular calcium handling. In endothelial cells, TMAO induces the release of IL-1b and IL-18 via inhibition of the SIRT3SOD2 pathway and activation of the NLRP3 inflammasome. It also promotes endothelial dysfunction by activating the PKC/NF-kB/VCAM-1 pathway. Collectively, these mechanisms ultimately contribute to the progression of heart failure.

All of the above preclinical and clinical studies seem to indicate that modest increases in plasma TMAO levels do not appear to have adverse effects on the circulatory system. Some scientists believe that TMA, rather than TMAO, is important in patients with higher cardiovascular risk, and future studies should evaluate both TMAO and TMA (Figure 5).

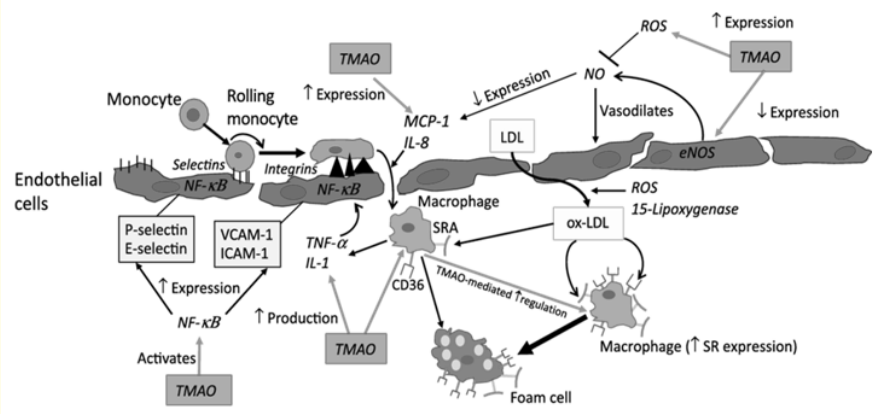

Figure 5: *Putative pro-inflammatory and pro-atherosclerotic actions of trimethylamine-N-oxide (TMAO). Pronounced elevations in TMAO to >10-20 μm, for example in advanced heart failure or chronic kidney disease, may be sufficient to modify multiple determinants of inflammation and atherosclerosis. Whether levels of circulating TMAO in obesity, diabetes or coronary heart disease are sufficient to significantly influence these processes is presently unclear. Symbols on the figure: CD36: Cluster of Differentiation 36; eNOS: Endothelial Nitric Oxide Synthase; ICAM-1: Intracellular Adhesion Molecule-1; MCP-1; Monocyte Chemoattractant Protein 1; NF-κB; Nuclear Factor κ-Light-Chain-Enhancer of Activated B Cells; ox-LDL: Oxidised-LDL; ROS: Reactive Oxygen Species; SR: Scavenger Receptor; SRA: Scavenger Receptor A: VCAM-1: Vascular Adhesion Protein-1 (Adapted from Naghipour S, Cox AJ, Peart JN., et al. [24]).*

Until recently, there was no direct evidence of the depolarizing effect of TMAO on the effector elements of the cardiovascular system. Glimpses of light at the end of the tunnel appeared recently, in 2020, when Oakley, Vallejo, Wang., *et al*. [25] hypothesized that pathological concentrations of TMAO would acutely increase cardiac and smooth muscle contractility and these effects may ultimately to contribute to cardiac dysfunction. Really, TMAO augmented contractility in isolated mouse hearts. Reverse perfusion of TMAO through the coronary arteries via a Langendorff apparatus also enhanced cardiac contractility.

In contrast, the precursor molecule, trimethylamine (TMA), did not alter contractility. Multiphoton microscopy, used to capture changes in intracellular calcium in paced, adult mouse hearts *ex vivo*, showed that TMAO significantly increased intracellular calcium fluorescence. Interestingly, acute administration of TMAO did not have a statistically significant influence on isolated aortic ring contractility. Oakley., *et al*. [25] concluded that TMAO directly increases the force of cardiac contractility, which corresponds with TMAO-induced increases in intracellular calcium but does not acutely affect vascular smooth muscle or endothelial function of the aorta.

Jaworska, Konor, Hutch., *et al*. [26] considered it controversial that trimethylamine oxide (TMAO), a product of hepatic oxygenation of trimethylamine (TMA) produced by intestinal bacteria, is a marker of cardiovascular risk. They noted that the mechanisms of increased concentrations and biological effects of TMAO are unclear, and the potential role of TMAO precursor, TMA, has not been investigated. They decided to evaluate the effect of age, as a cardiovascular risk factor, on plasma TMA and TMAO levels, intestinal bacterial composition, intestinal TMA penetration into the blood, and histological and hemodynamic parameters in 3-month-old and 18-month-old male rats. The cytotoxicity of TMA and TMAO was studied in human vascular smooth muscle cells. Old rats had a significantly different intestinal bacterial composition, significantly higher intestinal TMA penetration into the blood, and morphological and hemodynamic changes in

the intestine. *In vitro*, TMA at a concentration of 500 μmol/L (2-fold higher than in portal blood) reduced the viability of human vascular smooth muscle cells. In contrast, TMAO at a concentration 1000 times higher than physiological had no effect on the viability of human vascular smooth muscle cells. In conclusion, aged rats have higher plasma TMA levels due to the leaky gut. TMA, but not TMAO, affects the viability of human vascular smooth muscle cells.

Finally, they suggested that TMA, but not TMAO, may be a marker and mediator of cardiovascular risk. More research is clearly needed, and the effect of TMAO on blood vessels remains questionable.

Thus, increasing evidence suggests that the gut microbiota influences the cardiovascular system directly and indirectly through bioactive molecules. TMAO, a key metabolite produced by gut bacteria, is involved in the development of atherosclerosis and chronic endothelial dysfunction, but its effects on vascular tone, oxidative stress and inflammation remain poorly understood. Recent data [27] aimed to evaluate the acute effects of TMAO on vascular contractility in relation to markers of oxidative stress and inflammation. Aortic rings were obtained from laboratory rats and placed in a tissue bath system containing TMAO at concentrations of 300, 100, 10 μM. The level of isometric tension of smooth muscle was recorded under the action of phenylephrine and the endothelial-independent vasodilator sodium nitroprusside. Oxidative stress and inflammation were quantified by assessing NF-κB, NRF2, SOD1 and iNOS activity. After incubation of aortic rings in TMAO solutions for 1h, there was no difference in vasoconstrictor and non-endothelial vasodilator responses between the studied doses. TMAO acutely induced oxidative stress and inflammation by significantly increasing MDA levels and NF-κB, NRF2, SOD1 and iNOS expression, mostly in a dose-dependent manner.

This study showed no short-term effect of the studied TMAO doses on vascular contractility, but demonstrated an acute prooxidant effect and activation of major inflammatory pathways, which may partly explain the detrimental effects of TMAO on cardiovascular disease.

Thus, the cumulative inhibitory effect of TMAO ions on the outward current in vascular smooth muscle cells can lead to depolarization of cell membranes, a decrease in their excitability threshold. This creates of ideal conditions for the development of potential hypercontractility of smooth muscle cells and promotes an abnormally high increase in the level of tonic tension in the vascular wall in response to the appearance of a necessary external stimulus, for example, a decrease in the level of oxygenation.

It is important to recall that in experiments on isolated smooth muscles of the coronary arteries, they demonstrate a clearly pronounced rhythmic phasic activity. Unfortunately, we do not know for sure whether this phenomenon occurs in humans in real life conditions. It is possible that the presence of such an autorhythmic activity indicates the readiness of such an object for the development of tonic contraction and the development of spasm.

That is, under normal conditions, the normal reaction of the coronary circulation in response to a decrease in oxygenation is a moderate contraction of the large coronary arteries and dilatation of small vessels, arterioles. If the normal coordination in the work of large and small vessels in the myocardial blood supply system is disturbed, then events develop according to the worst scenario. Namely, the salutary relaxation of arterioles is possible only with the normal functioning of potassium channels and, above all, calcium-dependent potassium channels of high conductivity. An increase in the activity of C- and Rho-kinases, a violation of the functional activity of the endothelium, blockade of glycolysis and outward potassium current, individually or a combination of these factors in various combinations, makes this process uncontrollable and then a positive feedback is triggered with an inevitable lethal outcome.

Let us try to consider possible scenarios for the development of events in the development of coronary spasm. As you know, in order for clouds to form and rain to fall, condensation nuclei are needed in the atmosphere. For the formation of a spasmodic area in the wall of the coronary artery, there must be locus minor is resistance place in the wall of the vessel, i.e., the point or zone of least resistance in the chain of protection of the vascular wall from unwanted hypercontraction.

More precisely, there should be at least two such starting points. The first one must be localized in large epicardial arteries, and it really can be strictly local damage. This trigger is responsible for the abnormally strong contraction of the vascular wall in response to a vasoconstrictive stimulus. Such a hyperresponsive answer may be due to impaired integrity of the endothelium or a sharp decrease in the outward potassium conductivity in the membrane of smooth muscle cells. The second most important element of the scenario is a malfunction of metabolic processes that regulate the normal response of resistance arterioles to a decrease in the level of oxygenation, i.e. relaxation.

In other words, a bad scenario for the development of coronary spasm is when the resistance vessels do not respond with normal relaxation in response to a sudden cessation of arterial inflow. This can happen, for example, when glycolysis in the vascular smooth muscle cells of arterioles is somehow compromised, or the level of activity of C and Rho-kinases is abnormally increased in them. As a result, fatal arrhythmias and myocardial infarction occur.

Those, two events occurring simultaneously in different parts of the myocardial blood flow network should coincide in time and maximum their intensity. The probability of such a coincidence, fortunately, is not so great.

It is impossible to exclude the possibility of a very bad scenario for the development of events in coronary spasm, when the main events are played out at the level of large arteries. This is exactly the case where, according to the conclusion of Murphy's rule, if an event can develop according to the worst-case scenario, then it will develop exactly that way, and the pathologist will not see any signs of damage to the arteries indicating a possible cause of death. As in a well thought out and well-organized crime - there is a corpse, there are no traces of the criminal.

In this case, the coronary artery contracts so strongly and rapidly that the metabolism of the myocardial circulatory system does not have time to react. A sharp increase in pressure above the zone of occlusion provokes the development of a myogenic reaction and potentiates the constriction of smooth muscles. The pressure drops below the zone of occlusion reaches the critical pressure of closing the vessel and does not allow the development of salvage vasodilation.

What is the key (trigger) link of this chain of events in the coronary bed? This may be, for example, a violation of the integrity and functional activity of the endothelial lining in a certain section of the coronary network and, as a consequence, the direct effect of biologically active substances of a constrictor nature circulating in the blood directly on smooth muscle cells, bypassing the endothelium. That is, instead of endothelium-dependent relaxation, we will get a constrictor (endothelium-independent) reaction.

Perspectives of pharmacological correction

The question naturally arises: what can modern pharmacology offer to counteract PAF and TMAO? With PAF the situation is simpler. Ginkgolide B has been on sale for a long time. This is standardized extract of ginkgo biloba leaves EGb761^{R} and its anti-PAF efficacy was now clearly expressed in the treatment of depression, stress-induced disorders, pain, complications of diabetes, Parkinson disease, tinnitus and dizziness, reproductive dysfunction, etc. Current experimental and clinical data suggest that EGb761 (ginkgolide B) is a high-effective cytoprotective agent in cognitive disorders of various genesis, cardiovascular diseases (in the process of rehabilitation and as a medicine capable to reduce the volume of brain damage in stroke. In study Wang., *et al*. (2023) demonstrated the efficiency of ginkgolide B on blockade vascular remodeling after vascular injury [28].

Similar drugs are also needed to counteract excess TMAO. The registration of such a drug will not take much time because it is a food additive. We may be talking about the combination of beta-hydroxymethylbutyrate (β-hydroxyisovaleric acid) in the form of calcium salt and L-leucine that we propose. β-hydroxymethylbutyrate is a natural metabolite for the human body, which has the ability to improve lipid

and protein metabolism. There are studies that have shown a decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol in individuals with elevated total and LDL cholesterol levels, while individuals with normal cholesterol levels do not have this effect. In addition, by affecting the enzymes of the cell-signalling pathway (mTOR), this compound can contribute to the normalization of important cellular functions. The fact is that recently it has been established that the main factor of atherosclerosis is the formation under the action of microorganisms (microbiome) of the intestines of the substance trimethylamine, which, being absorbed into the blood and entering the liver under the influence of its enzymes, turns into the substance TMAO.

This hypothesis is based on structure-activity relationships. Hydroxymethylbutyrate (HMB) molecules are similar to 3,3-dimethylbutanol molecules, which are the most effective agents blocking TMA production in the gut microbiota [29]. *In silico* studies also support our hypothesis. Leucine acts in a similar way, as it is a precursor of HMB. It itself prevents the development of atherosclerosis and improves treatment outcomes in patients with heart attacks [30].

Here we should dwell in more detail on the role of short-chain fatty acids, and especially butyrate, in the vital activity of the intestinal microbiota. Let us recall that short-chain fatty acids (SCFAs) are a group of fatty acid compounds with an alkyl chain shorter than six carbons that includes butyrate, acetate, and propionate. SCFAs are found in both small and large intestines, except for butyrate, which is mostly located in the colon and cecum [31]. In passing through the intestinal epithelium, SCFAs interact with host cells, influencing the immune response. Their positive effects range from strengthening the intestinal barrier and supplying ample energy to the gut epithelial cells and to the microbiota. Additionally, they exert various functions on the physiology and immunity of the host, being considered metabolites with significant anti-inflammatory properties [32]. SCFAs turned out to be an inhibitor of histone deacetylase, promoting a tolerogenic and anti-inflammatory cellular phenotype essential for maintaining immunological homeostasis [33].

It is important to note that the dysbiosis commonly observed in inflammatory bowel diseases is linked to a reduction in activity of proinflammatory microbes such as adherent/invasive *E. coli* and H_2S producers. [34]. In addition, studies found a decrease in fecal SCFA levels in individuals with inflammatory bowel diseases [35], aligning with findings from quantitative PCR targeting the butyryl CoA:acetate CoA-transferase gene (primary mechanism for butyrate synthesis in the human microbiome).

Beta-hydroxybutyrate is chemically very similar to butyrate but is not synthesized in the intestine; instead, it is synthesized in the liver as the most important ketone body that provides energy under low-glycemic conditions and especially during periods of fasting or caloric restriction [36].

There is evidence to suggest that there is a specific axis in the body - gut-microbiota-brain and other organs, which affects pathophysiological processes in the body, i.e. butyrates should be considered as molecules modulating the effects of the gut-microbiota-internal organs axis [37].

Thus, TMAO gradually causes changes in kidney cells, vascular wall cells and immune response cells, which leads to damage to the vascular intima and cholesterol deposition. There are studies that show that β-hydroxymethylbutyrate helps reduce the formation of trimethylamine in the intestines and ensures the normalization of the complete composition of the microbiome, which as a result does not form (or forms significantly less) trimethylamine from precursors contained in food: choline, betaine, carnitine, lecithin, etc. Due to this, the concentration of trimethylamine in the blood and the possibility of its conversion to TMAO are reduced.

Blockade of flavin monooxygenase 3, a natural substance from cruciferous vegetables, by diindolylmethane, which ensures the conversion of TMA to TMA-O, can significantly enhance the positive effect of hydroxymethylbutyrate, as well as additional administration of leucine, which competes with carnitine. Therefore, a more pronounced effect should be inherent in a combination containing hydroxymethylbutyrate, diindolylmethane and leucine. Thus, we propose using a complex product for the prevention of atherosclerosis containing hydroxymethylbutyrate, diindolylmethane and leucine.

It is known that β-hydroxymethylbutyrate is used as a food product for special medical purposes. It improves muscle cell function, increasing the force of contractions, especially in the elderly and long-term immobilized people, people recovering from bone fractures, with debilitating diseases, including cachexia.

Diindolylmethane is a product of the body's processing of substances contained in broccoli and Brussels sprouts, which is able to reduce the activity of liver enzymes that convert trimethylamine to TMAO. It also exhibits antiatherogenic effects and is able to normalize estrogen levels, which has a positive effect on women in premenopausal and menopause, and also helps reduce the risk of prostate cancer in men.

Leucine is an indispensable proteinogenic amino acid that contributes to the development of muscle strength and endurance. It is a precursor to the formation of β-hydroxymethylbutyrate in the body, which makes it possible to prolong the effect of the latter. In addition, leucine can compete with carnitine for active transport into cells, contributing to a decrease in the latter's intake, which also reduces the possibility of formation of the trimethylamine metabolite from carnitine.

Thus, the above-mentioned dietary supplement is a source of β-hydroxymethylbutyrate, diindolylmethane and leucine. It is recommended for use by people whose diet includes meat products, people with an increased risk of developing atherosclerosis, people in a state of immobilization or recovering from bone fractures.

Conclusion

Unfortunately, there are no and cannot be any clear conclusions from this article. There will be none until we understand why phosphatidylcholine, the main building material of cell membranes, and trimethylamine, a product of normal intestinal microbiota activity, suddenly become factors that threaten the life of their host? True, there is nothing new in this situation. It is enough to recall the three main molecules that largely determine and regulate the vital activity of the human body - nitric oxide, carbon monoxide and hydrogen sulphide. Pharmacological targets have been identified for each of them. However, their range of action is from normal vasodilation to oxidative stress, vasoconstriction, apoptosis and cell death. As the ancients said, everything is medicine and everything is poison. The final effect depends on the concentration. Therefore, the reason lies in the disruption of the processes of regulation of the synthesis and secretion of these compounds. This is the first direction in which we need to work. However, there is another aspect of this problem. An abnormal reaction of the effector to a normal stimulus can also be explained by the fact that some undesirable changes have occurred in the effector elements themselves or in their environment. This could be hypoxia, a change in acidity, an excess of active forms of oxygen, atherosclerosis, etc. It is likely that fatal spasm of the coronary arteries develops due to an unfortunate coincidence in time of the action of these different factors.

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