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Original Article

Utilization of D-3-hydroxybutyrate by the isolated perfused heart under global no–flow ischemia and stress conditions

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إستخدام الموجات الصوتية في تشخيص خشونة مفصل الركبة الروماتيزمي

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الملخص العربي

في الظروف الاعتيادية يفضل القلب استخدام هيدروكسي بيوتريت عن الجلوكوز أو الاحماض الدهنية كمصدر للطاقة . يثبط هيدروكسي بيوتريت أكسدة الجلوكوز والأحماض الدهنية بينما الاجهاد الفسيولوجي وغير الفسيولوجي ينشط أكسدة الجلوكوز والأحماض الدهنية. الدراسة التالية تبحث في تأثير الإجهاد الكيميائي وغياب سريان الشريان التاجي على أكسدة هيدروكسي بيوتريت وإنتاج أسيتوأستيت في وجود وغياب محفزات ومثبطات مسارات الاشارات البيو كيميائية.

التأثير المحفز لمركب داينتروفينول على استهلاك هيدروكسي بيوتريت في القلب يتناسب طردي مع تركيز المحفز. غياب سريان الشريان التاجي وأينوماسين منشط CaMKK وأنيسومايسن منشط P38MAPKتحفز استهلاك هيدروكسي بيوتريت في القلب , المركبين STO-609 و PD-169316 يبطلان التأثير التحفيزي السابق.

الاستنتاج:
داينتروفينول منشط للمسارين AMPK و P38 MAPK في القلب وأنيسومايسن منشط P38MAPK وأينومايسن منشط CaMKK , جميعهم يحفزون استهلاك أو استخدام بيتاهيدروكسي بيوتريت في القلب, تثبيط المسارات السابقة AMPK و P38 MAPK و CaMKK في القلب يبطل التحفيز الناتج عن تنشيط مسارات AMPK و P38 MAPK في القلب. النتائج تشير إلى امكانية مساهمة AMPK و P38 MAPK أثناء تعرض القلب للإجهاد أو غياب الشريان التاجي في القلب وتنظيم أيض بيتاهيدروكسي بيوتريت وأن التأثير المحفز للاسكيميا على أيض هيدروكسي بيوتريت في القلب يتم عبر مسارات الاشارات البيو كيميائية لكل من AMPK و P38 MAPK

ABSTRACT

It is known that under normal conditions, D-3-hydroxybutyrate (D-3-HB) utilization is preferred over glucose and fatty acids by the isolated perfused heart. Moreover, D-3-HB inhibits oxidation whereas physiological and non-physiological stressors stimulate the oxidation of both major energy substrates. The following study investigates the effect of chemical stress and global no-flow ischemia-reperfusion on the utilization of D-3-HB and acetoacetate production in the isolated perfused rat heart, in the presence and absence of reported activators and inhibitors of some signaling pathways. The uncoupling of the oxidative phosphorylation of 2, 4-dinitrophenol (DNP) enhanced D-3-HB utilization in a concentration dependent manner. Global non-flow ischemia-reperfusion, and ionomycin and anisomycin, the activators of Ca2+calmodulin- dependent protein kinase kinase (CaMKK) and p38 mitogen-activated protein kinase (p38 MAPK), respectively, stimulate the utilization of D-3-HB. On the other hand,the inhibitors STO-609 and PD-169316 abolish ionomycin, anisomycin and global no-flow ischemia-stimulated utilization of D-3-HB. We conclude that: DNP [an activator of both cardiac AMP-activated protein kinase (AMPK) and p38 MAPK)], anisomycin (a selective activator of p38 MAPK), and ionomycin (a CaMKK activator) stimulate D-3-HB utilization in heart, and this stimulation is either partially or completely abolished by selective inhibitors of the above mentioned kinases. Our findings suggest the possible involvement of AMPK and p38 MAPK during stress in regulating D-3-HB utilization,, and that the ischemia-reperfusion stimulatory effect may possibly be mediated by the AMPK and p38 MAPK signaling pathways for further investigation.

Keywords

D-3-HB, Stress, AMPK, CaMKK, p38MAPK, Cardiac metabolism

INTRODUCTION

The heart primarily utilizes fatty acids, glucose and, ketone bodies as a sources of energy (1,2). Ketone bodies compete with glucose and fatty acids for utilization by the heart (3, 4, 5). D-3-hydroxybutyrate (D-3-HB) is not only an energy substrate but also plays a regulatory role in cardiac metabolism: it improves metabolic efficiency, decreases free radical damage, and presents cardioprotective effects (6). Fasting enhanced and chronic diabetes decreased D-3-hydroxybutyrate utilization in the isolated perfused rat heart (7, 8).

During exposure to such stressors as ischemia, ischemia-reperfusion, hypoxia, and anoxia, the activation of the AMP-activated protein kinase (AMPK) signaling pathwayplays an significant role in controlling cardiac metabolism. The stimulation of AMPK activity enhances catabolic pathways to provide the heart with ATP, and inhibits the consumption of AT,P since maintaining cellular ATP levels is vital for heart viability and function under stress related conditions (9, 10). The regulation of fatty acids and glucose metabolism in heart is mediated by the AMPK signaling pathway during physiological and non-physiological stress. Heart AMPK activation increases

glucose and fatty acid oxidation and inhibits protein synthesis (9,11, 12, 13, 14).

To the best of our knowledge, there has been no publications in the literature on the effect of ischemia and chemical stress on the utilization of D-3-hydroxybutyrate, and the role of the AMPK and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways in the regulation of D-3-hydroxybutyrate. The purpose of this investigation was to study D-3-hydroxybutyrate utilization under chemical stress and global no-flow ischemia, in the isolated perfused rat heart in the presence and absence of reported activators and inhibitors of some signaling pathways.. We used DNP as a chemical stressor to mimic hypoxia and to induce an energy demand in the heart.We also used ischemia-reperfusion to investigate the effect of metabolic stress on the utilization of D-3-hydroxybutyrate

MATERIAL AND METHODS

Animals

Normal male Albino Wister rats weighing between 250-350 g were used in the present study. Animals were housed under a constant room temperature of 22° C and a controlled light cycle (lights on between 06.00-18.00 h). The rats were fed ad libitum and had free access to food and water. The diet consisted of pellets containing 13% protein and 3% fat, manufactured by Grains and Flour Mills Organization, Jeddah, Saudi Arabia. All experiments were conducted in accordance with the guidelines of Umm Al- Qura University Council of Animal Care and were approved by the animal care committee of Umm Al-Qura Research Institute.

Chemicals

DL-3-hydroxybutyrate (sodium salt) (DL-3-HB), nicotinamide adenine dinucleotide (disodium salt, oxidized and reduced forms), D-3-hydroxybutyrate dehydrogenase (EC.1.1.1.30), acetoacetate (lithium salt)(Ac) , 2,4-dinitrophenol (DNP), Adenine 9-β-D-arabinofuranoside (Ara-A), 5-Iodotubercidin(Itub), 7-Oxo- 7-H-benzimidazo [2.1-] benz[de] isoquinoline-3-carboxylic acid - acetic acid (STO-609), Ionomycin(Iono), Pinacidil, PD-169316(PD), Anisomycin (Aniso), and Dimethylsulfoxid(DMSO) as vehicle (v), and all other chemicals maintained the highest available quality and were obtained from SIGM-ALDRICH, USA.

Media

The saline medium consisted of 142.2 mM sodium chloride and 0.5 mM sodium bicarbonate, and when equilibrated with atmospheric CO_2 at 4°C, had a pH of 7.4. The saline medium was used during the preparation of cannulation to cool the heart following excision.

Hearts were perfused for either two hours or 90 min with Krebs-Henseleit medium modified to contain half of the concentration of calcium and magnesium (MKHM), and oxygenated by equilibration with 5% CO_2 in oxygen. DL-3-HB was included at 5 mM corresponding to an initial concentration of the metabolically active D-3-HB of 2.5 mM. When the vehicle (v), dimethylsulfoxide (DMSO) was used, its concentration was no more than 0.2% v:v, and the addition of other substances are shown in the results and in the figure legends.

Perfusion Method

We utilized the Fisher and O'Brien(15) and Sultan (2) non-working heart perfusion technique to perfuse the rat hearts. This technique involves the continuous infusion of fresh media into a volume of recirculating perfusate that is kept constant by balanced withdrawal. Hearts (158) were removed from

fed rats under light diethyl ether anesthesia and placed in a cooled saline medium at 4°C and prepared for cannulation. Hearts were perfused with MKHM containing substance(s) at a pressure of 40 mmHg, and at an infusion rate of 30.65± 0.08 ml.hr^{-1} (158) for 120 or 90 min, and aperfusate temperature of 37°C. The perfusatewas passed through a 47 mm Millipore disc of 0.47 µm pore diameter (Millipore Corporation, Bedford, Mass, USA) supported by a Whatman No. 54 paper filter. Samples of perfusate were collected for five minutes, and alternate samples were analyzed. We collected twelve samples during the 2nd hour or six samples during the last 30 min of perfusion, respectively. Next, either six or three alternate fractions were used to determine the D-3-HB and Acetoacetate (Ac) concentrations.

Coronary flow of each perfused heart was measured at the 3rd minute (57.57±1.33 (118) ml.g.dry w^{-1}. min^{-1}) a successful preparation. Hearts with a coronary flow less than 30ml.g. dry w-^{1}. min-^{1} were suspected of being blocked and were disregarded(2).

Global no–flow ischemia protocol

Three groups of hearts were perfused with MKHM, containing DL-3-HB (5mM) in the absence and presence of either PD-169316 (1µM) or STO-609 (2.5µM) for 10 minutes.This served as the equilibration period of aerobic perfusion as coronary flow reached 60±1.61 ml. g.dry wt-1.min1 followed by 15 minutes of global no-flow ischemia. Then the perfusate flow through the heart was completely interrupted resulting in a subsequent 65 minute period of aerobic reperfusion with MKHM containing DL-3-BH (5mM) in the absence and presence of PD-169316 or STO-609. The coronary flow at the end of the reperfusion period was 63.71±2 ml. g.dry wt-1.min1 . Heart beats ceased between 2-4 minutes after stopping the flow of the perfusate and restarted after 1-2 minutes of reperfusion. On examining the heart at the end of perfusion period, the heart was soft, and there were no apparent ischemic patches.

Analytical methods

The estimation of D-3-hydroxybutyrate (D-3-HB) and acetoacetate (Ac) were performed by the methods of Williamson and Mellanby (16) and Mellanby and Williamson, respectively.

Calculation and expression of the results

In the steady state condition, the concentrations of the substrate in successive fractions were not measurably different. The second hour of the perfusion period represents the steady state period. During this period, the following equation was used to estimate the rate of D-3-hydroxybutyrate utilization and acetoacetate production:

$$u = i(a - x)/w$$

Where u: the rate of utilization (negative if production), µmoles.g.drywt^{-1}.h^{-1}

i: the rate of infusion, ml.h^{-1}

a: the initial perfusate concentration, mM

x: the perfusate concentration, mM

w: the dry weight of the heart, g

The difference between the rate of D-3-HB utilization and the rate of acetoacetate production is assumed to be the rate of D-3-HB oxidation, and can be calculated.

Results were expressed as a mean of individual experiments ±SEM and the number of observations given in parentheses.

Statistical analysis

Comparisons between groups were assessed by the two-tailed Student's t-test for independent observations using Texasoft, WINKS SDA Software, 6th Edition, Cedar Hill, Texas, 2007.

RESULTS

The effect of DNP on D-3-hydroxybutyrate utilization

To establish the effect of DNP on D-3-HB utilization, six groups of hearts were perfused with MKHM containing DL-3-HB (5mM) and different concentrations of DNP. The control group did not contain DNP while the other groups contained DNP at the following concentrations: 5, 10, 25, 50, or 100 µM. The rate of D-3-HB utilization and acetoacetate production during the 2nd 60 minutes of perfusion is shown in Figure 1. DNP stimulated D-3-HB utilization by 23%, 34%, and 40% at 10, 25, and 50 µM, respectively ($p \geq 0.001$). Regarding the heart rate, no significant effect was observed at 5 µM, whereas at 100 µM, heart beats were irregular, and the preparation was unstable (four experiments results are not shown).We considered that DNP at 50 µM had a maximum effect on D-3-HB utilization without any deterioration of the perfused heart preparation; therefore, all subsequent experiments were performed at this concentration.

Figure 1. The effect of dinitrophenol on the rates of D-3-HB utilization and acetoacetae production.

Hearts were perfused with MKHM containing DL-3-HB (5mM) in the absence (control) and presence of dinitrophenol (DNP; 5, 10, 25, or 50 µM) for 120 min as described in the methods section. Results indicate means ± SEM and the number of individual observations are given in parentheses. The rates of D-3-HB utilization (white bar) and acetoacetate production (black bar), were estimated during the steady state and refer to the last 60 min of the perfusion period. $^{*}p \leq 0.001$ DNP vs control.

NB: DL-3-HB (5mM), should be present under every bar but it is as shown in figure for simplicity

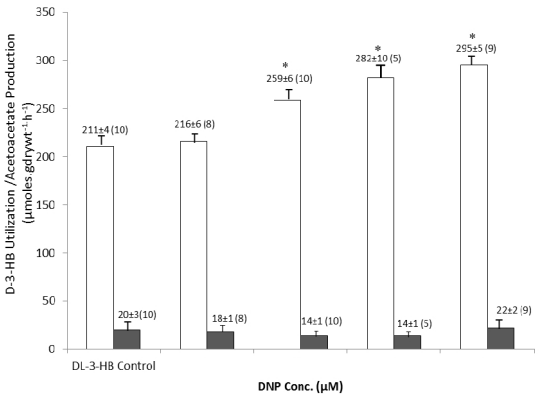

Fig. 1. The effect of dinitrophenol(DNP) on the rates of D-3-HB utilization and acetoacetate production

The stimulatory effect of DNP on the utilization of D-3-HB is concentration dependent, and there is no significant effect of DNP on acetoacetate production. Almost 90-95% of D-3-HB is not recovered as acetoacetate, and it is assumed to be oxidized and provides the heart with energy in the presence and absence of DNP.

The effect Ara-A and Iodotubercidin

DNP activates AMPK in cardiomyocytes, and Ara-A inhibits DNP-stimulated AMPK phosphorylation and glucose uptake (12). Iodotubercidin (Itub) inhibits adenosine kinase in neonatal hearts (18), basal AMPKα2 activity in skeletal muscle (19), and decreases AICAR- and cyanide-stimulated glucose uptake in heart papillary muscles (13). We tested the effect of two inhibitors on the action of DNP in stimulating D-3-HB utilization.

Two groups of hearts were perfused withMKHM containing DL-3-HB (5mM)

and vehicle (v) in the presence and absence of DNP (control), and another four groups of hearts were perfused with MKHM containing DL-3-HB, vehicle, and either Ara-A (1mM) or Itub (5µM), in the presence and absence of DNP. Figure 2 shows that both substances Ara-A and Itub inhibited the basal rates of D-3-HB utilization by 26% and 23% $p \leq 0.005$ and $p \leq 0.001$, respectively, and without significant effect on the rate of acetoacetate production in the absence of DNP. Ara-A partially inhibited rates by 8% $p \leq 0.02$, and Itub abolished DNP-stimulated D-3-HB utilization without significant effect on acetoacetate production. The vehicle (DMSO) had no significant effect on the utilization of D-3-HB and acetoacetate production in the presence and absence of DNP.

Figure 2. The effect of Ara-A and iodotubercidin on the rates of D-3-HB utilization and acetoacetate production in the presence and absence of dinitrophenol.
Hearts were perfused with MKHM containing DL-3-HB (5mM), vehicle (v), and either with or without dinitrophenol (DNP; 50µM) for 90 min as described in the methods section. The addition of either Ara-A (1mM) or iodotubercidin (itub; 5µM) is also shown in the figure. Results indicate means ± SEM and the number of individual observations are given in parentheses. The rates of D-3-HB utilization (white bar) and acetoacetate production (black bar), were estimated during the steady state and refer to the last 30 min of the perfusion period.
$^{*}p \leq 0.005$ Ara-A vs control, $^{**}p \leq 0.001$ Itubvs control, DNP+Itubvs DNP control, $^{***}p \leq 0.02$ DNP+Ara-Avs DNP control.
NB: DL-3-HB (5mM) and vehicle (v), should be present under every bar but it is as shown in figure for simplicity

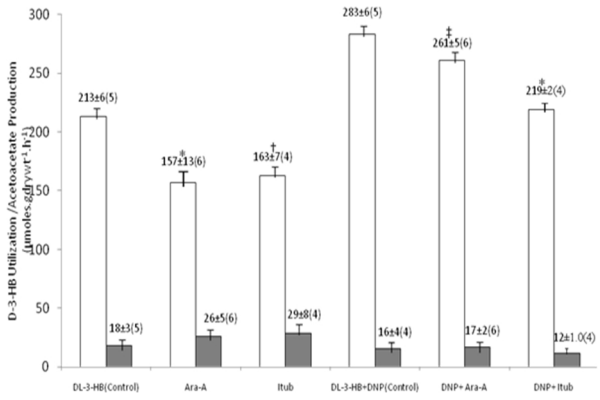

Fig. 2. The effect of Ara-A and iodotubercidin(Itub) on the rates of D-3-HB utilization and acetoacetate production in the presence and absence of dinitrophenol

The effect of Ionomycin and STO-609

The calcium ionophore,ionomycin, increased the cytosolic calcium concentration (20) and activated AMPK phosphorylation by the upstream kinase is Ca^{2+}calmodulin-dependent protein kinase kinase (CaMKK).This activation was inhibited by STO-609 (21, 22, 23), and DNP also induced an elevation of cytosolic Ca^{2+} (24, 25, 26). We used ionomycin to activate AMPK through the upstream kinase, CaMKK.
Two groups of hearts were perfused with MKHM containing DL-3-HB (5mM), vehicle, and ionomycin (1.3µM) in the presence and absence of STO-609 (2.5 µM). Figure 2 shows ionomycin stimulated the rate of D-3-HB utilization by 28% $p \leq 0.001$, and almost all of the utilized D-3-HB was fully oxidized. STO-609 abrogated the ionomycin-stimulated D-3-HB utilization and increased the rate of acetoacetate production to about 9% of the of the utilized D-3-HB $p \leq 0.005$. Another two groups of hearts were perfused with MKHM containing DL-3-HB, vehicle, and DNP in the presence and absence of STO-609. Figure 3 shows that STO-609

partially inhibits DNP-stimulated D-3-HB utilization by 16% p≤0.001, and the production of acetoacetate is increased to about 21% p≤0.001 of the utilized D-3-HB. In hearts perfused with MKHM containing DL-3-HB, vehicle, and STO-609, STO-609 had no effect on both the basal rate of D-3-HB utilization and on acetoacetate production.

Figure 3. The effect of ionomycin, STO-609, and pinacidil on the rates of D-3-HB utilization and acetoacetate production.
Hearts were perfused with MKHM containing DL-3-HB (5mM) and vehicle (v) for 90 min as described in the methods section, with the addition of ionomycin (iono; 1.3 µM), STO-609 (2.5 µM), pinacidil (100 µM) or dinitrophenol (DNP; 50 µM) to the perfusate as also shown in the figure. Results indicate means ± SEM and the number of individual observations are given in parentheses. The rates of D-3-HB utilization (white bar) and acetoacetate production (black bar) were estimated during the steady state and refer to the last 30 min of the perfusion period. $^{*}p \leq 0.001$ Ionovs control, Ionovs Iono+STO-609, for D-3-HB utilization, and DNP+STO-609 vs DNP control, for Ac production. **p≤0.005 Ionovs Iono+STO-609 for Ac production.
NB: DL-3-HB (5mM) and vehicle (v), should be present under every bar but it is as shown in figure for simplicity

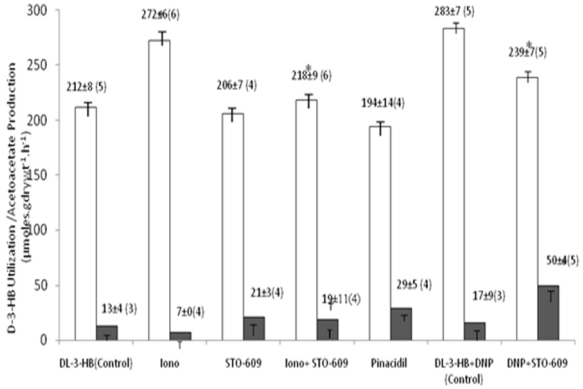

Fig. 3. The effect of ionomycin (Iono) STO-609 and pinacidil on the rates of D-3-HB utilization and acetoacetate production

The effect of Pinacidil

Pinacidil is a K_{ATP} opener, and DNP can acts as K_{ATP} opener (27, 28). Pinacidil and DNP activate sarcolemmal K_{ATP} channels in cardiomyocytes (29). AMPK mediates preconditioning in cardiomyocytes by regulating sarcolemmal ATP-sensitive K^{+} channels (30). We used pinacidil to investigate whether K_{ATP} channels could be involved in mediating the effects of DNP.

A group of hearts was perfused with MKHM containing DL-3-HB, vehicle, and Pinacidil(100 µM). Figure 3 shows that pinacidil had no effect on the basal rate of D-3-HB utilization.

The effect of Anisomycin and PD-169316

Anisomycin activates cardiac p38 MAPK in a time- and dose-dependent manner without affecting AMPK (31), and PD-169316 is a selective inhibitor of p38 MAPK (12, 32). DNP stimulates p38 MAPK activity, and PD-169316 inhibits this effect in cardiomyocytes (12). We used anisomycin to activate p38 MAPK, which mimics the ischemia-reperfusion condition(44,45,46).
Two groups of hearts were perfused with MKHM containing DL-3-HB, vehicle, and anisomycin (10µM) in the presence and

absence of PD-169316 (1µM). Figure 4 shows that anisomycin stimulated the rate of D-3-HB utilization by 26% p≤0.001, and PD-169316 fully abolished anisomycin-stimulated D-3-HB utilization. Another two groups of hearts were perfused with MKHM containing DL-3-HB, vehicle, and DNP in the presence and absence of PD-169316. Figure 4 shows that PD-169316 statistically had no significant effect on DNP-stimulated D-3-HB utilization. In hearts that were perfused with MKHM containing DL-3-HB, vehicle, and PD-169316, PD-169316 had no effect on the basal rate of D-3-HB utilization. Each of these groups presented acetoacetate production percentage ratesat around 8% of the utilized D-3-HB.

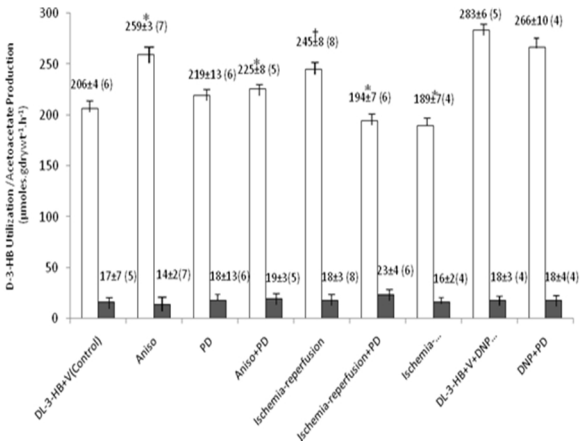

Fig. 4. The effect of anisomycin(Aniso), PD-169316 ,STO-609 and global ischemia reperfusion on the rates of D-3-HB utilization and acetoacetate production. vehicle (v)

Figure 4. The effect of anisomycin, PD-169316, and global ischemia-reperfusion on the rates of D-3-HB utilization and acetoacetate production.
Hearts were perfused with MKHM containing DL-3-HB (5mM) and vehicle (v), as described in the methods section, with the addition of anisomycin (aniso; 10 µM), PD-169316 (PD; 1 µM), or dinitrophenol (DNP; 50 µM) to the perfusate as also shown in the figure. Global ischemia-reperfusion was induced as described in the methods section. Results indicate means ± SEM and the number of individual observations are given in parentheses. The rates of D-3-HB utilization (white bar) and acetoacetate production (black bar) were estimated during the steady state and refer to the last 30 min of the perfusion period.*p ≤ 0.001 Anisovs control, AnisovsAniso+PD, ischemia vs ischemia +PD, Ishemiavs Ischemia+STO-609. **p≤0.002 Ischemia vs control.
NB: DL-3-HB (5mM) and vehicle (v), should be present under every bar but it is as shown in figure for simplicity

<u>The effect of global no-flow ischemia, PD-169316 and STO-609</u>

Hearts were subjected to no-flow ischemia for 15 min and allowed to equilibrate for 10 min, followed by a period of reperfusion for 65 min.The utilization of D-3-HB was estimated during the last 30 min of the reperfusion period. The rate of D-3-HB utilization was increased by 19% p≥0.001 during the reperfusion period as shown in Fig.4. The oxidation rate of D-3-HB was 93% of the utilized D-3-HB. In another two groups of hearts subjected to no-flow ischemia, the addition of either PD169316 (1µM) or STO-609 (2.5µM) to the circulation media abolished the ischemia-reperfusion-stimulated utilization of D-3-HB.

DISCUSSION

DNP stimulates the utilization of D-3-HB in the isolated perfused heart in a concentration-dependent manner. This stimulatory effect is due to either one or more of the multi effects of DNP. DNP is an uncoupler and causes

mitochondrial membrane deplorization, a reduction in the mitochondrial membrane potential (33, 34), a decrease in myocyte $NADH^+$ (26), a raise in the cytosolic concentration of Ca^{2+} and increases the AMP/ATP ratio (24, 25, 34). DNP stimulates the activity of AMPK, p38 MAPK and the uptake of glucose in cardiomyocytes (12). Either Ara-A or iodotubercidin decreased the basal rate of D-3-HB utilization, indicating a possible role for AMPK in normal conditions. Ara-A caused partial inhibition of the DNP-stimulated D-3-HB utilization, whereas iodotubercidin abolished the DNP-stimulated D-3-HB utilization indicating the role of AMPK in stressful conditions. The partial inhibition of Ara-A on DNP-stimulated D-3-HB utilization could be due to Ara-A inhibiting the AMPK α_2 isoform (19), which is also predominant in cardiac cells (35, 36). DNP activates both the α_1 and α_2 isoforms of AMPK (37). Therefore, the DNP stimulatory effect could be a result of the activity of the AMPK α1 isoform. The effect of iodotubercidin could be due to the inhibitiory effect on adenosine kinase (18), adenosine transport (38), AMPK α_2 (19) and AMPKα1 (39). Ara-A partially inhibits DNP-stimulated AMPK and glucose uptake in cardiomyocytes (12), and iodotubercidin partially inhibits cyanide-stimulated glucose uptake in heart papillary muscle (13). This is consistent with our findings since Ara-A and Itub inhibit basal D-3-HB utilization.

The inhibition of basal D-3-HB utilization by both Ara-A and Iodotubercidin (selective inhibitors of AMPKα2), the partial and complete inhibition of DNP-stimulated D-3-HB utilization by Ara-A and iodotubercidin, respectively, and DNP's ability to raise the cytosolic Ca^{2+} concentration and the sensitivity of AMPKα2 to AMP drove researchers to modulate the upstream kinase CaMKK. CaMKK activates AMPK (21, 22, 40), particularly AMPKα1 (41), in response to the rise of cytosolic Ca^{2+} independently of the AMP/ATP ratio (42), and DNP increases cytosolic Ca^{2+} (24, 25, 26). Ionomycin,aCaMKK activator, stimulated D-3-HB utilization and was then abolished by STO-609, a selective inhibitor of CaMKK (23). This supports the involvement of AMPK in regulating D-3-HB utilization, moreover; it indicates the role of calcium as an intracellular messenger in D-3-HB metabolism. STO-609 partially inhibited DNP-stimulated D-3-HB utilization, indicating that the DNP effect is partially mediated by increasing the level of cytosolic Ca^{2+}, further activating CaMKK. These findings support the possibility that the Ca^{2+} - CaMKK - AMPK signaling pathway mediates DNP-stimulated D-3-HB utilization.

DNP could also activate K_{ATP} channels by depolarizing the intra-mitochondrial membrane and altering the Ca^{2+} cytosolic concentration (27, 28). Pinacidil opens K_{ATP} channels and (29) has no significant effect on the basal rate of D-3-HB utilization, therefore, data do not support that enhancing K_{ATP} channels mediates DNP-stimulated D-3-HB utilization.

The role of p38 MAPK

DNP activates p38 MAPK and AMPK in cardiomyocytes (12), and PD-169316 is a selective inhibitor of cardiac p38 MAPK (32, 12). Anisomycin potently activates cardiac p38 MAPK without stimulating the phosphorylation of AMPK and AKt (31, 43). Anisomycin mimics ischemia-reperfusion in activating p38 MAPK (44, 45, 46). Our finding that anisomycin stimulated D-3-HB utilization and that PD-169316 abolished this stimulation, strongly support the involvement of the p38 MAPK signaling pathway in the regulation of D-3-HB metabolism independent of the AMPK signaling

pathway. We also demonstrated that PD-169316 had no significant effect on both basal D-3-HB utilization and DNP-stimulated D-3-HB utilization; although DNP activated p38 MAPK and PD-169316 partially inhibited p38 MAPK in cardiomyocytes (12). Therefore, we were unable to conclude that the DNP effect on D-3-HB utilization is mediated through the p38 MAPK signaling pathway. However, we did not exclude the involvement of p38 MAPK in D-3-HB regulation since anisomycin-activated D-3-HB utilization is abolished by PD-169316.

In this study, we demonstrated that modulating either the AMPK or p38 MAPK signaling pathways affects D-3-HB utilization. The stimulation and inhibition of the AMPK/p38 MAPK signaling pathways were associated with the stimulation and inhibition of D-3-HB utilization, respectively.

Mimicking either chemical hypoxia or ischemia-reperfusion is associated with the enhancement of the utilization of D-3-HB in the heart. Ischemia activates AMPK and p38 MAPK, and the activation of p38 MAPK occurs independently of AMPK (47). PD-169316, an inhibitor of p38 MAPK, and STO-609, an inhibitor of CaMKK, cancel ischemia-reperfusion stimulated D-3-HB utilization. This supports the idea that ischemia-reperfusion-stimulated D-3-HB utilization is mediated by p38 MAPK and AMPK signaling pathways. Stress that alters the AMP/ATP ratio, mitochondrial and cytosolic concentration of calcium modulates D-3-HB metabolism. Ionomycin and anisomycin modulate AMPK and p38 MAPK, respectively, without affecting the AMP/ATP ratio, and both modulators stimulate D-3-HB utilization.

Pelletier and Coderre (4) reported that prolonged pretreatment of cardiomyocytes for 16h with D-3-HB caused the partial inhibition of DNP-stimulated AMPK and abolished stimulation by DNP of p38 MAPK.D-3-HB also partially inhibited DNP-stimulated glucose uptake in cardiomyocytes. They suggested that the inhibitory effect of D-3-HB on glucose uptake is due to the inhibitory effect of D-3-HB on AMPK / p38 MAPK activities, but they did not estimate D-3-HB utilization. It is possible that D-3-HB can limit its own utilization under certain conditions by inhibiting AMPK/p38 MAPK activities, serving as a feed-back control. However, we did not estimate AMPK/p38 MAPK activities, and the perfusion period did not exceed 2h. Therefore, we would not expect to record the effect of D-3-HB on both kinases. The citrate level is increased in hearts perfused with D-3-HB (48), and it known that citrate inhibits AMPK phosphorylation in rat hypothalamus (49), suggesting a possible mechanism whereby D-3-HB inhibits AMPK. Moreover, acetoactate increases the phosphorylation of extracellular signal-regulated kinase ½ (ERK1/2) and p38 MAPK in hepatocytes (50), and we found that DNP stimulates acetoacetate utilization in the heart (unpublished observation). It is conceivable that D-3-HB protects the cells against ATP depletion by providing NADH to the electron transport chain. D-3-HB may either limit or counteract the effect of DNP (decrease in mitochondrial membrane potential and ATP/ADP ratio) by providing a high supply of reducing equivalents to the respiratory chain, since it is oxidized directly in the mitochondrial matrix. D-3-HB further compensates for the energy deficit caused by either DNP or metabolic stress. DNP contributes to proton leakage, which keeps the cell NAD^+/NADH ratio sufficiently high and allows the required carbon metabolism to continue . It has been proposed that the high NAD^+/NADH ratio enhances AMPK activity

(51), but others findings lack any sufficient support (52).

Fasting (24 or 48h) is associated with the stimulation of cardiac D-3-HB utilization (8). AMPK is activated by fasting and is suppressed by Ara-A (53). Also, Ara-A decreased AMPK phosphorylation in the heart in response to the deprivation of glucose (54). Taken together, these findings showed that the modulation of either the AMPK or p38 MAPK signaling pathways had a role in regulating D-3-HB utilization. Our study also showed that DNP stimulated D-3-HB utilization in the isolated perfused heart is mediated by the AMPK signaling pathway, and that Ca^{2+} has a significant role in regulating D-3-HB utilization through its activation of CaMKK, which then activates AMPK. Both mechanisms, altering either the ATP/AMP or Ca^{2+} levels, could independently contribute to the enhancement of D-3-HB utilization.

We proceeded to observe that mimicking chemical hypoxia with DNP, or chemical ischemia–reperfusion with anisomycin, or activating AMPK/p38 MAPK stimulated the utilization of D-3-HB, and the inhibition of either kinases are associated with the reduction of D-3-HB utilization in the isolated perfused heart. AMPK and p38 MAPK are stimulated in response to ischemia (9, 31, 45). No-flow ischemia stimulates AMPK α2 and α1 activities and is most likely mediated by LKB1 (AMPKK) and CaMKK, respectively (41), and AMPK α2 has a greater dependence on AMP (55). Cardiac AMPK activity is stimulated by ischemia and reperfusion (11). Since it is well known that such stressor effects are mediated by AMPK/p38 MAPK, our data supports the idea that both kinases are involved in the regulation of D-3-HB metabolism in the heart. Since PD-169316 and STO-609 abolished D-3-HB utilization stimulated by ischemia–reperfusion, our data also support that both AMPK and p38 MAPK mediate the ischemia-reperfusion effect. We encountered a few limitationsthat include having not measured either the AMPK or p38 MAPK activities, and using the available selective activators and inhibitors of the both kinases.

We conclude that: DNP (an activator of both cardiac AMPK and p38 MAPK), anisomycin (a selective activator of p38 MAPK), and ionomycin (a CaMKK activator), stimulate D-3-HB utilization in heart, and this stimulation is either partially or completely abolished by selective inhibitors of the above mentioned kinases. The chemical stress may be possibly mediated by changes in Ca^{2+} concentration and /or AMP/ATP levels, through the AMPK/p38 MAPK signaling pathways. Global no-flow ischemia stimulates D-3-HB utilization possibly through the same signaling pathways. However, this needs further investigations to elucidate the involvement of the above mentioned signaling pathways.

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CONFLICT OF INTEREST

Nothing to declare.

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