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Elevated adropin: A candidate diagnostic marker for myocardial infarction in conjunction with troponin-I

Suna Aydin[a,b], Tuncay Kuloglu[c], Suleyman Aydin[d,*], Mehmet Kalayci[e,f], Musa Yilmaz[d], Tolga Çakmak[e,f], Mehmet Nesimi Eren[g]

[a] Department of Cardiovascular Surgery, Elazig Research and Education Hospital, Elazig 23100, Turkey
[b] Firat University, School of Medicine, Department of Anatomy, Elazig 23119, Turkey
[c] Firat University, School of Medicine, Department of Histology and Embryology, Elazig 23119, Turkey
[d] Firat University, School of Medicine, Department of Medical Biochemistry (Firat Hormones Research Group), Elazig 23119, Turkey
[e] Laboratory of Medical Biochemistry, Elazig Research and Education Hospital, Elazig 23100, Turkey
[f] Department of Cardiology, Van Ercis State Hospital, Van, Turkey
[g] Dicle University, School of Medicine, Department of Cardiovascular Surgery, Diyarbakir 21280, Turkey

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ABSTRACT

Myocardial infarction (MI; "heart attack") can cause injury to or death of heart muscle tissue (*myocardium*) owing to prolonged ischemia and hypoxia. Troponins and CK-MB are released from heart muscle cells during MI. It has been demonstrated that energy expenditure is regulated by adropin expressed in the endocardium, myocardium, and epicardium. We hypothesized that adropin is released into the bloodstream during myocardial muscle injury caused by MI, so the serum level rises as myocytes die. Therefore, we examined the association between adropin expression and myocardial infarction in isoproterenol-induced myocardial infarction. Rats were randomly allocated to six groups. After treatment they were decapitated and their blood and tissues were collected for adropin measurement. Changes in adropin synthesis in rat heart, kidney and liver tissues in isoproterenol (ISO)-induced MI were demonstrated immunohistochemically. Serum adropin concentrations were measured by ELISA, and troponin-I, CK and CK-MB concentrations by autoanalysis. The results demonstrated that cardiac muscle cells, glomerular, peritubular and renal cortical interstitial cells, hepatocytes and liver sinusoidal cells all synthesize adropin, and synthesis increased 1–24 h after MI except in the liver cells. The findings elucidate the pathogenesis of MI, and the gradual increase in serum adropin could be a novel diagnostic marker and serve as an alternative to troponin-I measurement for diagnosing MI.

Introduction

Cardiovascular disease (CVD) remains the most common cause of death and disability in both developed and developing countries [11]. The category includes congestive heart disease, high blood pressure, and myocardial infarction ("heart attack"), which results from a reduction of coronary blood flow extensive enough to make the oxygen supply to myocardial tissues insufficient [8,24]. Ischemia (restriction in blood supply) and consequent oxygen shortage, if left untreated for long enough, can cause myocardial cell death and necrosis [4,7]. Myocardial infarction (MI) is currently diagnosed by integrating the history of the presenting illness, prolonged chest pain, "silent infarct", painless infarct, a typical electrocardiogram (ECG) pattern involving the development of pathological Q waves, and a rapid rise and fall of CK-MB and the typical rise and gradual fall of troponin-I [4,7]. Troponin-1 was first described as a biomarker specific for acute myocardial infarction (AMI) in 1987 [10] and is now the biochemical "gold standard" for diagnosing AMI according to a consensus of the American College of Cardiology (*ACC*) and European Society of Cardiology (*ESC*) [1,28]. *Early diagnosis* is *vital* if irreversible heart tissue injury is to be avoided [4,7,28]. Therefore, we need new early-appearance, accurate, precise, readily accessible, cost-effective cardiac markers for better diagnosis and prognosis of MI. Several million patients annually seek care in emergency, cardiology and cardiovascular surgery departments when chest pain or other symptoms suggest an acute coronary syndrome (ACS), but only ~10% are subsequently confirmed to have AMI [21].

A new metabolic hormone, adropin, was isolated in 2008 by Kumar et al. from liver and brain [19] and could be linked with

* Corresponding author. Tel.: +90 533 4934643; fax: +90 424 2379138.
E-mail address: saydin1@hotmail.com (S. Aydin).

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AMI, since in addition to pancreatic, liver, brain and kidney tissues, adropin synthesis was demonstrated immunologically in the endocardium, myocardium, and epicardium [6]. After adropin is released into blood, it plays various roles, such as nitric oxide bioavailability, apoptosis and energy homeostasis, heart failure and a novel predictor of coronary atherosclerosis [reviewed, 5, 22, 33]. Some researchers have also noted a positive correlation between plasma adropin levels and flow-mediated dilatation values [31]. Adropin levels are also significantly lower in patients with cardiac syndrome X (CSX) than healthy subjects, and lower serum adropin is an independent risk factor for CSX [9,reviewed, 5].

On the basis of the foregoing information, we hypothesized that the adropin synthesized in the endocardium, myocardium, and epicardium [6] could serve as a new biological marker for diagnosis and prognosis of myocardial ischemia, because injury to heart muscle cells is likely to release adropin into the bloodstream. In order to test our hypothesis, AMI in rats was experimentally induced by isoproterenol [1-(-3,4-dihydroxyphenyl-2-isopropyl amino ethanol) hydrochloride], a synthetic catecholamine and adrenergic agonist that causes severe stress in the myocardium resulting in infarct-like necrosis of the heart muscle [25]. Impaired cardiac function is also detrimental to the kidney (cardiorenal interaction) in clinical [3] and experimental [27] settings, and AMI causes significant abnormality in liver function [30]. Therefore it is important to check renal and liver adropin expression in the experimental model of MI induced by isoproterenol in rats. However, no study to date has reported the fate of adropin in ISO-induced AMI. Hence, the first aim was to compare adropin expression in cardiac muscle, kidney and liver tissues in rats with ISO-induced MI against controls immunohistochemically. The second aim was to measure ISO-induced changes in serum creatine kinase (CK), myocardial muscle creatine kinase (*CK-MB) and* troponin-I concentrations by autoanalysis and adropin concentrations by ELISA.

Materials and methods

All protocols of animal experiments used for this study accorded with the principles set out by the Institutional Animal Ethics Committee (FUIAC; issue: 2013-4-69) at our university and with the policy of the European convention for the protection of vertebrate animals. All animals were acclimatized for one week before the experiment began, being fed a standard pellet diet and given water ad libitum. The rats (2.5 months old) were divided into six treatment groups of six animals each: group I (control), group II (1 h), group III (2 h), group IV (4 h), group V (6 h), and group VI (24 h). The rats were housed in cages in an animal facility with a 12-h dark/light cycle and controlled temperature and humidity. Water and food were administered unrestricted throughout the study. MI in groups III, IV, V, and VI was induced by single subcutaneous injections of ISO (200 mg/1000 g body weight) dissolved in normal saline. Isoproterenol (ISO) hydrochloride (cat. no. I5627) was purchased from Sigma Chemical Co., St. Louis, MO, USA. This ISO dose and injection route is known to cause significant alterations in biochemical parameters and also moderate heart tissue necrosis [18]. At the end of the experimental periods, the rats were decapitated under ketamine–HCl (75 mg/kg) and 10 mg/kg xylazine–HCl anesthesia and serum adropin levels and tissue adropin immunoreactivity were measured. To check whether MI had occurred, histological sections were prepared and examined by light microscopy to assess gross myocyte injury, and serum levels of CK, CK-MB and troponin-I, which are superior biochemical markers of MI, were measured. Other relevant details of experimental MI were described previously [2,18]. Cardiac, renal and liver tissues were resected, cleaned for immunohistochemistry (IHC) washed in ice-cold saline, fixed in 10% formaldehyde and embedded in paraffin. Blood samples were divided into two aliquots, one for classical biochemical parameters and the other for adropin measurement. Blood was collected in plain biochemical tubes containing 500 KIU aprotonin to protect from proteolysis, and centrifuged at 4000 rpm (1792 × *g*) at RT for 5 min. Sera were collected and stored at −80 °C until adropin levels were measured.

Masson staining

After one day in 10% formaldehyde, the blocks were dehydrated and embedded in paraffin, cut into 4–5 μm slices, heated overnight in a 60 °C incubator, and then dewaxed and stained with Masson dye. One slice was chosen from each rat and examined under a microscope. This Masson trichrome staining was used for histological evaluation. Histological injury scores were allotted according to a previously published classification method (0: absence, +1: weak, +2: medium, +3: strong), noting the increments of inflammatory cells, congestion, fibrosis, edema, disruption of tissue integrity and necrosis [18]. The overall tissue injury score was calculated from 10 randomly selected microscopic fields in four to six individual sections per heart using a camera attached to an Olympus B ×50 microscope.

Immunohistochemistry

The avidin–biotin-peroxidase complex (ABC) was used for immunohistochemistry as per Hsu et al. [16] with minor modifications [18]. All procedures were followed exactly as described previously except for the primary antibody step. Briefly, paraffin-embedded 4–5 μm sections were cut, deparaffinized and passed through a graded alcohol series. They were incubated in citrate buffer A (pH 6.0) and heated in a microwave oven (750 W; 7 min + 5 min) to recover antigenicity, then for 1 h with the primary antibody. The adropin primary antibody was diluted 1/400 (anti-adropin antibody, ab12800 Abcam, Cambridge, UK), applied, and incubated in a humid environment for 60 min at RT. As a control for antibody specificity, the primary antibody step was omitted. The primary antibody was highly specific against the proteins under examination and did not cross-react with related molecules. There were positive reactions when the sections were incubated with the substrate 3-amino-9-ethylcarbazole (AEC) + AEC chromogen (AEC Substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC, Lab Vision Corporation, USA) and counterstained with Mayer's hematoxylin, then covered with lamellae to make permanent preparations. These preparations were examined under a light microscope and photographed using an attached camera (Olympus B × 50, Tokyo, Japan). Immunohisochemical staining was scored for both intensity and prevalence on a scale of 0 to +3 (0: absence, +1: weak, +2: medium, +3: strong).

Serological measurements

Serum adropin was measured using commercial ELISA kits (cat no: EK-032-35) and procedures (Phoenix Pharmaceuticals, Belmont, CA, USA). The lowest detectable concentration of adropin was 0.1 ng/mL, with intra- and inter-assay variations of 10 and 15%, respectively. Sample absorbance at 450 nm was measured with an ELX 800 ELISA reader. Serum CK and CK-MB levels were measured by autoanalysis (Advia 1800 Chemistry System, Siemens Healtcare Diagnostics Inc. Tarrytown, NY, USA) using Advia Chemistry commercial kits, and serum troponin-I concentration by chemiluminescence using a Siemens IMMULITE 2000 XPi Immunoassay System (Siemens Healtcare Diagnostics Inc. Flanders NJ. USA) and commercial kits (Siemens Healtcare Diagnostics Products Ltd. Llanberis, United Kingdom).