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Regulation of liver cell glucose homeostasis by dehydroabietic acid, abietic acid and squalene isolated from balsam fir (*Abies balsamea* (L.) Mill.) a plant of the Eastern James Bay Cree traditional pharmacopeia

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ABSTRACT

In our previous study, *Abies balsamea* (L.) Mill., a plant used in Cree traditional medicine, had a strong effect on the regulation of glucose homeostasis in liver cells. This study aimed to isolate and identify its active constituents using a bioassay-guided fractionation approach as well as to elucidate their mechanism(s) of action. The effect of the crude extract and its constituents was evaluated on the activity of Glucose-6-Phosphatase (G6Pase) and Glycogen Synthase (GS) and phosphorylation of three kinases, AMP-activated protein kinase (AMPK), Akt and Glycogen Synthase Kinase-3 (GSK-3). Three compounds, abietic acid, dehydroabietic acid and squalene, were isolated from the most active fraction in the bioassays (hexane). The compounds were able to decrease the activity of G6Pase and to stimulate GS. Their effect on G6Pase activity involved both Akt and AMPK phosphorylation with significant correlations between insulin-dependent and -independent pathways and the bioassay. In addition, the compounds were able to stimulate GS through GSK-3 phosphorylation with a significant correlation between the signaling pathway and the bioassay. Dehydroabietic acid stood out for its strongest effect in all the experiments close to that of the crude extract. These compounds may have potential applications in the treatment of type 2 diabetes and insulin resistance.

1. Introduction

Type 2 diabetes, the most common type of diabetes, is caused by decreased insulin sensitivity in target organs like liver, muscle and adipose tissue, and a deficiency in insulin secretion (Cheng et al., 2009). Aside from reduced peripheral glucose disposition (mostly skeletal muscle uptake), increased hepatic glucose production is a major contributor to diabetic hyperglycemia. Insulin regulates the expression of genes encoding main enzymes implicated in hepatic gluconeogenesis and glycolysis (Pilkis and Granner, 1992). It inhibits the transcription of genes encoding fructose-1,6-biphosphatase and Glucose-6-Phosphatase (G6Pase) mainly through the activation of the kinase Akt and the phosphorylation of some transcription factors, like forkhead transcription factor O1 (FoxO1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha), involved in the expression of these enzymes (Puigserver et al., 2003; Yoon et al., 2001).

Glycogen Synthase Kinase-3 (GSK-3) inhibits Glycogen Synthase (GS) through phosphorylation. The insulin pathway activates GS through phosphorylation and inhibition of GSK-3 and activation of protein phosphatase 1 (PP1). G6Pase is the rate-limiting enzyme of gluconeogenesis and GS is the enzyme responsible for glucose storage as glycogen in liver and muscle. Therefore, both enzymes control hepatic glucose production and were evaluated in this study as markers for potential antidiabetic activity.

AMP-activated protein kinase (AMPK), key regulator of insulin-independent pathways, is also involved in hepatic glucose homeostasis. It is considered to be the target of Metformin, the most commonly used oral hypoglycemic drug worldwide. Once

Abbreviations: AMPK, AMP-activated protein kinase; CREB, c-AMP-regulator element-binding protein; EtOAc, ethyl acetate fraction; FoxO1, forkhead transcription factor O1; G6Pase, Glucose-6-Phosphatase; GS, Glycogen Synthase; GSK-3, Glycogen Synthase Kinase-3; Hexane, hexane fraction; HNF4, Hepatic Nuclear Factor 4; H_2O, aqueous fraction; MeOH, methanol fraction; PEPCK, phospho-enol pyruvate carboxykinase; PGC-1alpha, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PP1, protein phosphatase 1; PPAR gamma, peroxisome proliferator-activated receptor gamma.

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phosphorylated, AMPK is activated and leads to the inhibition of gluconeogenesis and stimulation of glucose storage as glycogen (Rui, 2014; Viollet et al., 2007). Both insulin-dependent (Akt) and -independent pathways (AMPK) are thus involved in regulating hepatic glucose production.

According to the International Diabetes Federation (IDF), 382 million people are living with diabetes; this number will reach 592 million in 2035. Eighty percent of diabetic people are living in low- and middle-income countries and most of them are aged between 40 and 59 (IDF, 2013). Aboriginal populations are at higher risk to develop type 2 diabetes than the general Canadian population (Ekoe et al., 1990; Hegele, 2001).

A screening study of seventeen anti-diabetic plants used by the Eastern James Bay Cree (Canada) showed that *Abies balsamea* (L.) Mill. (Pinaceae), enhanced glucose transport in muscle cells and adipocytes (Spoor et al., 2006). Another screening study on the same seventeen plants showed that *A. balsamea* stood out for marked effects on glucose homeostasis in liver cells. It decreased G6Pase activity, involving both Akt and AMPK phosphorylation, and simultaneously stimulated GS activity by inhibiting GSK-3 (Nachar et al., 2013). In continuity, we now sought to elucidate the active principles responsible of the antidiabetic effect of *A. balsamea* using a bioassay-guided fractionation and to evaluate their effect on key enzymes of gluconeogenesis and glycogen synthesis as well as key kinases regulating these enzymes.

2. Results and discussion

2.1. LDH test (cytotoxicity)

H4IIE and HepG2 cells were treated overnight (16–18 h) with the crude extract of *A. balsamea*, its solvent fractions, HPLC peaks and pure compounds at various concentrations and the LDH released was measured. Maximal non-toxic concentrations for each sample were used for all subsequent experiments (results shown in Table 1 of supplementary data).

2.2. Effect of A. balsamea and its fractions on glucose production and storage in liver cells

It was recently shown that *A. balsamea* significantly reduced G6Pase activity and strongly stimulated GS (Nachar et al., 2013). Indeed, impaired hepatic glucose production and storage are two important factors contributing to the pathogenesis of the obesity-diabetes disease continuum (Hutton and O'Brien, 2009; Wang and Roach, 1993). These previous results prompted further work in order to isolate potential active fractions and principles and to evaluate their effect on hepatic glucose homeostasis in cultured hepatocytes. G6Pase was selected because it is the rate-limiting enzyme for gluconeogenesis (Schmoll et al., 2000), whereas GS plays the same role for glucose storage (Wang and Roach, 1993). As observed previously (Nachar et al., 2013), *A. balsamea* crude extract, tested at 50 μg/mL, exerted an inhibitory effect on G6Pase (48% decrease) close to that of the positive control, insulin, (56% reduction), tested at its supraphysiological concentration (100 nM). When this crude extract was fractionated using solvents of increasing polarity, bioactivity was associated primarily with the hexane fraction (50% inhibition), whereas EtOAc and MeOH showed intermediate inhibition (23%) and the aqueous fraction had a non-significant effect on G6Pase activity (13% inhibition) (Fig. 1A). All the fractions were tested at 25 μg/mL according to their maximal non-toxic concentration.

In terms of GS activity, the MeOH fraction was without effect whereas the aqueous and EtOAc solvent fractions activated GS to an extent similar to that of the positive control (activation by 2-fold compared to DMSO, Fig. 1B). In contrast, *A. balsamea* crude extract and its hexane fraction stood out with 14- and 50-fold activation, respectively compared to DMSO.

The results of both assays (G6Pase and GS) confirmed that the hexane fraction exhibited the most important effect, halfing G6Pase activity and stimulating GS 50-fold. Therefore, further fractionation of the hexane extract was carried out, guided by G6Pase and GS activity assays.

2.3. Bioassay-guided subfractionation of the hexane fraction

Preparative scale isolation of the hexane fraction yielded eight isolates (PK1–PK8) listed in Table 1 (compound identification). LC–MS analyses were developed and applied to identify diterpenes that are known to be predominantly present in the non-polar fraction of coniferous plants including *A. balsamea* (Saleem et al., 2010). Our plant metabolomics MS library of over 200 compounds was used with a special focus on resin acids previously isolated and reported to be present in *A. balsamea* cones and bark (Guerrero-Analco et al., 2010; Hall et al., 2013). The presence of dehydroabietic acid (**2**), abietic acid (**1**) and squalene (**3**) was confirmed based on the similarity of protonated molecular ions and the fragments (elution at 11.0 min, 12.0 min and 12.7 min, respectively). Besides the three resin acids mentioned PK3, PK7 and PK8 showed protonated molecular ions and similar fragments as dehydroabietic acid and were putatively assigned as derivatives of dehydroabietic acid.

When tested in the G6Pase bioassay, seven of the eight peaks demonstrated varied yet statistically significant inhibitory potential that ranged from 14% to 51% when compared to vehicle control (0.1% DMSO; 0% inhibition reference; Fig. 2A). Similarly, six out of the eight subfractions stimulated GS activity 1.5- to 2.5-fold compared to vehicle control (0.1% DMSO; 100% activation reference; Fig. 2B). In both assays, PK1, PK2, PK3, PK4 and PK5 exerted biological effects on the activity of G6Pase and GS that were generally closer to the insulin positive controls than the three others. This led us to further characterize these five peaks.

According to the LC–MS/MS analysis, these peaks, albeit distinct, showed some similarities in their constituents. We found that most of them contained the three major compounds above **1**, **2** and **3**. They all belong to the terpene family, which includes a large number of organic compounds found in variety of plants and tress. Terpenoids are considered as multifunctional natural compounds. Indeed, compound **1** exhibited many beneficial effects like an anti-inflammatory effect *in vivo* (Fernandez et al., 2001) and in macrophages *in vitro* (Takahashi et al., 2003), regulation of lipid metabolism (Takahashi et al., 2003), and treatment of allergic reactions (Ulusu et al., 2002). A recent *in vivo* study showed that **1** has an anti-obesity effect in mice fed a high-fat diet and does this by regulating adipogenesis (Hwang et al., 2011). Similarly, **2** also regulates inflammation in macrophages and adipocytes (Kang et al., 2008), activates peroxisome proliferator-activated receptor gamma (PPARgamma) and stimulates glucose uptake in adipocytes (Takahashi et al., 2011). Furthermore, an *in vivo* study showed that **2** improves diabetes and hyperlipidemia in obese diabetic KK-Ay mice (Kang et al., 2009). For its part, **3**, an isoprenoid, has been reported to be an oxygen scavenging agent (Saint-Leger et al., 1986) and to have antitumor properties and immune enhancing activities (Newmark, 1997; Reddy and Couvreur, 2009) (see Fig. 3).

2.4. Identification of dehydroabietic acid (2) as the most active compound acting on key enzymes of hepatic glucose production

According to the literature, none of the compounds, isolated from the most active peaks, had been tested for actions on hepatic glucose homeostasis. The study here showed that **1** and **3** similarly