Egg-derived ACE-inhibitory peptides IQW and LKP reduce blood pressure in spontaneously hypertensive rats

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ABSTRACT

Two angiotensin converting enzyme (ACE) inhibitory tripeptides, IQW and LKP, were previously characterized from egg white protein ovotransferrin. ACE is a key enzyme of the renin–angiotensin system (RAS) which generates angiotensin II (Ang II) from its precursor and increases blood pressure (BP) in the body. This study tested the blood pressure lowering potential of orally administered IQW and LKP in spontaneously hypertensive rats. IQW and LKP treatment decreased mean blood pressure (MAP) by ~19 and ~30 mmHg, respectively, compared to untreated SHRs. The change in BP was accompanied by the preservation of nitric oxide dependent vasorelaxation and lowering of plasma Ang II levels. Furthermore IQW, but not LKP, also reduced intercellular adhesion molecule-1 (ICAM-1) expression and nitrotyrosine levels in arteries, suggesting additional protective effects against inflammation and oxidative/nitrosative stress. These results demonstrate anti-hypertensive effects of IQW and LKP in vivo and a reduction of circulating Ang II levels, with additional anti-inflammatory and antioxidant effects mediated by IQW.

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1. Introduction

Functional foods and nutraceuticals have gained considerable attention due to increasing evidence of their beneficial effects on treatment and management of chronic diseases. Food derived bioactive peptides with potential health benefits are one of the major areas of recent research (reviewed in Shahidi & Zhong, 2008). These peptides are generally latent in their parent proteins but exhibit physiological benefits over and above their...
expected nutritional value only after release through enzymatic digestion, fermentation, or food processing (Kitts & Weiler, 2003; Rutherford-Markwick & Moughan, 2005). Among various food derived bioactive peptides, antihypertensive peptides with angiotensin converting enzyme (ACE) inhibitory properties are of research interest due to the high prevalence of hypertension (Tibazarwa & Damasceno, 2014). Hypertension is a major risk factor for developing cardiovascular diseases (CVDs) which is a key cause of global morbidity and mortality (Danaei et al., 2013). Chronically elevated blood pressure (BP) level at or above 140/90 mmHg is defined as hypertension (Chockalingam, 2008). A number of pharmacological drugs have been used in the management of hypertension. Many of these drugs require lifelong adherence to therapy; yet many patients still have poorly controlled BP and suffer adverse side effects (Khanha, Lefkowitz, & White, 2008; Moser & Franklin, 2007). Functional foods and/or nutraceuticals are derived from natural sources and generally considered safe; hence these have become potential alternatives to synthetic pharmacological drugs.

Multiple food derived factors can significantly reduce high BP (Hermansen, 2000; Zarraga & Schwarz, 2006). It has been suggested that partially replacing dietary carbohydrate with protein may also help in the prevention and treatment of hypertension (Rebolz et al., 2012). The pathophysiology of hypertension is complex, although the crucial roles of renin–angiotensin system (RAS), oxidative stress, and vascular inflammation in the development and the persistence of hypertension have been determined (Opalil & Haber, 1974; Paravicini & Touyz, 2002; Rahman, Gilmour, Jimenez, & MacNee, 2002; Salavemini, Ischiropoulos, & Cuzzocrea, 2003; Stauffer, Westby, & DeSouza, 2008). Bioactive peptides from food protein hydrolysis have been known to exhibit antioxidant, anti-inflammatory, and antithrombotic activities along with ACE inhibitory effects (Balti et al., 2012; Fujita, Sasaki, & Yoshikawa, 1995; Ichimura et al., 2009; Miguel & Alexandre, 2006). Therefore, a multifunctional peptide with ACE-inhibitor, antioxidant, and anti-inflammatory activities could be an ideal candidate for the prevention and management of hypertension.

Eggs are an economically valuable source of dietary protein (Sumner et al., 2011). In addition to their well-known nutritional value, egg is also a rich source of bioactive peptides. Ovotransferrin, an iron binding glycoprotein, contributes ~13% of the total protein in egg white (Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982). Our earlier study had identified three tripeptides IRW (Ile-Arg-Trp, molecular weight: 473.58 Da), IQW (Ile-Gln-Trp, molecular weight: 445.51 Da) and LKP (Leu-Lys-Pro, molecular weight 356.46 Da) from enzymatic digestion of the ovotransferrin with in vitro angiotensin converting enzyme (ACE) inhibitory properties (Majumder & Wu, 2010). Further studies also demonstrated that some of these peptides have significant antioxidant and anti-inflammatory properties in cultured vascular endothelial cells (Majumder, Chakrabarti, Davidson, & Wu, 2013a). Another publication from our group has further characterized the in vivo antihypertensive effect of IRW and its possible mechanisms in a rat model of hypertension (Majumder et al., 2013b). Spontaneously hypertensive rat (SHR) is a well-recognized animal model that develops hypertension at an early age (~12–14 weeks) and remains hypertensive throughout their lives (Dornas & Silva, 2011; Trippodo & Frohlich, 1981). To test novel anti-hypertensive therapies and to study the pathophysiology of hypertension SHRs have been widely used in scientific research (Bagnot et al., 2010; Cutts & Kim, 2010; Katayama et al., 2008; Nakamura, Naramoto, & Koyama, 2013). Despite the in vitro evidence, a lack of in vivo data may impede the further development of IQW and LKP as viable anti-hypertensive options. Therefore, the present study evaluates the in vitro efficacy and likely mechanisms of action of IQW and LKP in a well-characterized animal model of hypertension.

## 2. Materials and methods

### 2.1. Peptides

Both IQW and LKP were chemically synthesized and supplied by GenScript Inc. (Piscataway, NJ, USA), and their purity (>98%) was verified by HPLC-MS/MS.

### 2.2. Animal model

The animal experimental procedures were similar to those used in our previously published study on the effects of ovotransferrin derived tripeptide IRW in SHRs (Majumder et al., 2013b; Majumder, Panahi, Kaufman, & Wu, 2013c). Briefly, 12–14 week old male SHR animals (270.0 ± 10.5 g) were obtained from Charles River (Senneville, QC, Canada). They were kept in the University of Alberta animal facility for at least 1 week to undergo acclimatization. During the acclimatization and experimental phases SHR animals were exposed to a 12 hour (light:dark) cycle, in a humidity and temperature-controlled (23 °C) quiet room. Standard rat chow (0.3% NaCl) and water ad libitum were given to all the rats. All procedures were approved by the University of Alberta Animal Welfare Committee (Protocol # 611/09/10/D) in accordance with the guidelines issued by the Canada Council on Animal Care.

### 2.3. Experimental design

After acclimatization, 13–15 week old SHR animals were surgically implanted with telemetry transmitters (PA-C40; Data Science International Minneapolis, MN) for BP monitoring in live animals. Following 7–10 days of recovery period after surgery, the animals were randomly assigned to 3 treatment groups – untreated (control), IQW (15 mg /kg BW) and LKP (15 mg /kg BW). The doses were selected based on a previously published in vivo study on the egg derived peptide IRW (Majumder et al., 2013b, 2013c). On the first day of recording (day 0) all the animals received 20 mL of Ensure (Abbott Nutrition, St. Laurent, QC, Canada). From day 1 onwards, the peptides (IQW and LKP) were dissolved in Ensure for palatability and administered once per day continuously up to 18 days. Untreated animals were given Ensure alone as a vehicle control. BP was recorded for a 24 h period (10 s of every 1 min) on days 0 (baseline), 3, 6, 9, 12, 15 and 18 under the conditions described earlier. The animals were then sacrificed by exsanguination via excision of the heart under inhaled isoflurane anesthesia, at the end of the experimental period. The blood was collected from the heart and transferred immediately into ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Vacutainer, NJ, USA) and centrifuged
monoclonal antibody, BD Biosciences, San Jose, CA, USA were normalized to β-actin (anti-β-actin rabbit polyclonal antibody, Abcam Inc., Toronto, ON, Canada) and expressed as a fold change compared to untreated samples. Samples from all treatment groups were loaded on the same gel for quantitative analysis. Anti-β-actin was used at 0.5 μg/mL, while eNOS, ICAM-1 and VCAM-1 antibodies were used at 1 μg/mL. Secondary goat-anti-rabbit and donkey-anti-mouse antibodies (Li-Cor Biosciences, Lincoln, NB, USA) were used to visualize the bands in a Li-Cor Odyssey Biolmager and quantified by densitometry with corresponding software (Odyssey V3.0, Li-cor Biosciences).

2.8. Plasma biomarker analysis

Blood plasma samples were collected, centrifuged (1000 × g for 20 min at 4 °C) and stored at −80 °C for further analysis. Angiotensin II (Ang II) was quantified by ELISA kits (Ang II ELISA, Cayman Chemical, Ann Arbor, MI, USA) as per the manufacturers’ instructions.

2.9. Immunofluorescence

Aortic sections were prepared for immunostaining as described previously (Majumder et al., 2013b). On the day of experiment, tissue sections were fixed with acetone, blocked in 1% bovine serum albumin (BSA) and incubated with rabbit polyclonal antibody against nitrotyrosine (dilution 1:500; Chemicon, Temecula, CA, USA), overnight at 4 °C. The secondary antibody (dilution 1:200; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) incubation was done in the dark for 30 min. Vectashield H-1200 Mounting Kit, containing the nuclear stain DAPI (Vector Laboratories, Burlington, ON, Canada), was used as a mounting media with glass cover-slips and the tissue sections were visualized immediately under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Metamorph imaging software (Molecular Devices, Sunnyvale, CA) was used to capture the images at 200× magnification. To ensure a good representation at least three different images were taken from each tissue section. To detect any nonspecific binding, a control image from a tissue section treated with secondary antibody alone was used in each slide. The fluorescence intensity for each image was then calculated by subtracting the background fluorescence intensity from the respective control image, as described in our previous study (Majumder et al., 2013b). The mean fluorescence intensity was then calculated for quantitative analysis.

2.10. Statistics

All data presented are mean ± SEM of 4–6 animals for each treatment group. Two-way ANOVA was used to determine the interaction between two factors (time and treatment), while a Bonferroni’s post-hoc test used to compare the BP differences between different groups. For vascular function (MCh curve) data, nonlinear regression method was used for curve fitting, and one-way ANOVA followed by Bonferroni’s post-hoc test or unpaired t-test was used to determine the maximum response (Emax) values as necessary. ICAM-1, VCAM-1 and eNOS bands, nitrotyrosine levels in tissue as well as Ang II levels in the plasma were analyzed by using one-way ANOVA. A $p$ value <0.05 was considered statistically significant.
3. Results

3.1. IQW and LKP treatments do not alter body and organ weights

No significant change in body weight was observed by the peptide treatment (Figure 1A). The average body weights of the animals at the end of the study (day 18) in the SHR untreated group, IQW treated group and LKP treated group were 369.17 ± 4.9, 359.06 ± 4.1 and 366.2 ± 4.13 g, respectively. No significant differences were observed in the liver, kidney and heart weights (Figure 1B, C and D) at the end of the experiments between different treatment groups.

3.2. IQW and LKP administration reduce blood pressure in SHRs

After 18 days of IQW and LKP treatment SBP was significantly decreased in both IQW and LKP groups to 161.40 ± 1.6 mmHg and 152.23 ± 0.8 mmHg respectively, compared to the untreated value of 182.43 ± 3.0 mmHg (Figure 2A). MAP and DBP with both IQW and LKP treatment also showed similar effects. The MAP and DBP for the untreated group were 168.49 ± 2.1 and 142.79 ± 2.9 mmHg, compared to 144.20 ± 2.0 and 121.13 ± 1.6 mmHg for the IQW treatment group, and 137.81 ± 1.0 and 117.32 ± 2.8 mmHg for the LKP treatment group, respectively, after 18 days of treatment (Figure 2B and C). With both peptides, significant blood pressure reduction was observed initially on day 12 and the trend continued to the end of the study period (day 18). However, no significant change was observed in HR (Figure 2D) among the groups.

Circadian variations or nocturnal dipping of blood pressure (MAP, SBP and DBP) were calculated from the mean BP during each 12 h light cycle (light/dark). The circadian variations of BP were disturbed in the untreated animals during the light and dark cycles. Despite the reductions in BP, the impaired circadian variation in BP (typically observed in SHRs with established disease) was not restored by IQW or LKP treatment (Figure 3A–D).

3.3. IQW and LKP restore nitric oxide (NO) mediate vasorelaxation

Response to phenylephrine (FE) vessel constriction in the mesenteric arteries was unaffected in the IQW and LKP treatment groups compared to the untreated group (data not shown). MCh induced vasodilatation was significantly enhanced in both the
3.4. IQW and LKP decrease plasma Ang II levels

The anti-hypertensive effects of IQW and LKP were associated with changes in circulating levels of Ang II in plasma. Ang II levels in the untreated group were $23.96 \pm 1.7 \text{ pg/mL}$ compared to $12.42 \pm 0.7 \text{ pg/mL}$ and $12.62 \pm 1.04$ in the IQW and LKP treated groups, respectively (Figure 6). These results suggest that both these peptides (IQW and LKP) can inhibit ACE in vivo and thus decrease the production of circulating Ang II in SHR plasma.

3.5. IQW and LKP treatment alters inflammatory markers and oxidative/nitrosative stress

IQW treatment significantly decreased the expression of the pro-inflammatory adhesion molecule, ICAM-1 without affecting levels of VCAM-1, whereas LKP did not alter the expression of either ICAM-1 or VCAM-1 in mesenteric arteries (Figure 7A and B).

Similarly, a significant decrease in nitrotyrosine staining (a marker of increased peroxynitrite generation, and, hence increased oxidative/nitrosative stress) in aortas of IQW treated
animals was observed but nitrotyrosine levels did not alter after LKP treatment (Figure 8). These results suggest that IQW can also act as an anti-inflammatory and antioxidant agent and thus make an additional contribution to ameliorate cardiovascular disease conditions.

Fig. 3 – IQW and LKP cannot restore the circadian rhythms of BP in SHRs. (A, B and C) SBP, DBP and MAP (mmHg) values from untreated SHRs or those treated with IQW or LKP were recorded during light and dark cycles over 18 days. (D) Summary graphs to demonstrate the effects of peptides (IQW and LKP) on circadian rhythm in MAP. Data represented as mean ± SEM from n = 4–6 animals per treatment group.

4. Discussion

Results from the present study demonstrated that egg derived bioactive tripeptides IQW and LKP significantly reduced blood pressure in adult male SHRs. Furthermore, both IQW and LKP restored NO mediated vasorelaxation and reduced plasma Ang II levels. Finally, IQW but not LKP treatment attenuated the expression of pro-inflammatory adhesion molecule (ICAM-1) and oxidative/nitrosative stress.

Given the global burden of hypertension and associated CVDs, results from this study may provide a food derived option for management of hypertension and generate a template to develop anti-hypertensive nutraceuticals with these peptides. Various peptides of different food origins have proven beneficial against CVDs, such as peptides with anti-hypertensive (ACE inhibitory), cholesterol lowering, anti-thrombotic, anti-inflammatory, and antioxidant activities (Balti et al., 2012; Turpeinen et al., 2009). Furthermore, food derived peptides exhibit multiple bioactive functionalities which can counteract the disease process at multiple points in its pathophysiological development (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Meisel, 2004). Therefore, it is apparent that food-protein derived bioactive peptides can exhibit regulatory functions above and beyond their basic nutritional value. IQW and LKP are two ACE inhibitory peptides previously characterized from the egg white protein ovotransferrin (Majumder & Wu, 2010). Moreover, IQW also exhibited anti-inflammatory and antioxidant properties in cultured human vascular endothelial cells (Majumder et al., 2013a). Given this background, we evaluated the antihypertensive effects of IQW and LKP in animals after oral administration.

SHR is an animal model of human essential hypertension, which demonstrates persistent high BP along with increased RAS activity, increased oxidative stress, and pronounced inflammatory response. Hence, SHRs are widely used to validate the efficacy of new antihypertensive agents (Trippodo & Frohlich, 1981). Results from the present study show that BP can be significantly reduced after treatment with IQW or LKP. Both the peptides could decrease BP without any significant changes in HR. Therefore, the peptides appear to preserve the normal cardiac responses which would likely minimize the risk of developing arrhythmias and other related complications.

The reduction of blood pressure after IQW and LKP treatments are concomitant with the reduction in plasma Ang II levels at the end of the study. Ang II as a vasoconstrictor is a key contributor in the RAS pathway which regulates BP and plays a key role in the pathophysiology of hypertension (Bader & Ganten, 2008; Oparil & Haber, 1974; Peach, 1977). While we previously determined the ACE inhibitory effects of both IQW and LKP (Majumder & Wu, 2010), LKP was also identified from bonito fish protein by Yokoyama, Chiba, and Yoshikawa (1992). The same group has also investigated the effect of LKP in vivo via intravenous administration where it reduced blood pressure by ~18 mm Hg in 4 h in hypertensive animals (Fujita & Yoshikawa, 1999). Moreover, a longer lasting effect was reported for LKPNM, a pro-drug type ACE inhibitor of LKP. However, this may not be the sole mechanism by which these peptides modulate blood pressure in this study.
The vascular function studies revealed that both IQW and LKP restored the nitric oxide (NO) mediated vasorelaxation. This suggests that these treatments could increase the bioavailability of NO; resulting in increased relaxation (Ramzy et al., 2006; Salvemini et al., 2003; Yang, Huang, Kaley, & Sun, 2009). Interestingly, neither of these peptides increased eNOS expression, suggesting the restoration of NO mediated vasorelaxation may be mediated by increasing NO bioavailability. These findings are in contrast to our previous results with IRW where a combination of ACE inhibitory, anti-inflammatory and antioxidant effects were observed to reduce BP (by ~40 mm Hg) in the same animal model (Majumder et al., 2013b).

An increased level of reactive oxygen species (ROS) often contributes to the pathology of hypertension (Hong, Hsiao, Cheng, & Yen, 2001; Pennathur & Heinecke, 2007; Salvemini et al., 2003). ROS such as superoxide (O$_2^\cdot$) can hinder the bioavailability of NO by generation of peroxynitrite (—ONOO$^-$), a highly reactive species that leads to a pro-inflammatory phenotype through tyrosine nitration of various proteins. Earlier studies have shown that a reduction in —ONOO and tyrosine nitration in SHRs, brought about by inhibiting ROS can reduce blood pressure (Cabassi et al., 2001; Hong et al., 2001). Similarly, our study showed that IQW but not LKP treatment can significantly reduce nitrotyrosine levels in aorta, suggesting an antioxidant effect of IQW. In addition, IQW also reduced the expression of the inflammatory adhesion molecule ICAM-1. The latter finding is in accordance with our previous study showing an antioxidant and anti-inflammatory effect of IQW on human endothelial cells (Majumder et al., 2013a).

In addition, LKP also appears to enhance NO sensitive vasorelaxation although no effects on eNOS expression and NO scavenging (by nitrotyrosine staining) could be found, suggesting a novel mechanism of action possibly through modulation of vascular reactivity at the level of endothelial derived hyperpolarizing factors (EDHF) or endothelin pathways (Jakala et al., 2009; Maes et al., 2004) which could be a target for future research. In contrast, IQW not only acts as an ACE inhibitor but also exerts anti-inflammatory and

Fig. 4 – IQW and LKP administration restore the nitric oxide dependent vasodilatation in mesenteric arteries of SHRs. (A) IQW and LKP significantly increased maximal vasorelaxation to MCh. (B, C and D) Addition of L-NAME (100 μM) prior to MCh treatment attenuated vasorelaxation in (C) IQW-treated and (D) LKP-treated but not in the (B) untreated groups. Data represented as mean ± SEM from n = 4–6 animals per treatment group. ** indicates P < 0.01 compared to the untreated group.
antioxidant effects, suggesting its multifactorial action on blood pressure. Thus, our present study shows that peptides initially identified as ACE inhibitors (through in vitro studies) may also exhibit in vivo antihypertensive effects through additional mechanisms. The observed differences in antioxidant and anti-inflammatory properties of the two peptides can be attributed to their constituent amino acids. For example, IQW contains tryptophan (W) at its C-terminal, an amino acid known to have antioxidant and anti-inflammatory effects (Huang, Majumder, & Wu, 2010; Suhas & Gowda, 2012; Zamfirova et al., 2014). Indeed short peptides (3–5 amino acids) with W at their C-terminal were identified as potent antioxidants in a QSAR study (Li & Li, 2013). On the other hand, peptides with hydrophobic amino acids at both N- and C-terminals are predicted to inhibit ACE (Wu, Aluko, & Nakai, 2006). As both IQW and LKP satisfy this requirement, it is not surprising that both exert ACE inhibitory effects.

In conclusion, the in vivo anti-hypertensive effects of orally administered IQW and LKP appear to be mediated through similar pathways involving increased NO mediated vasodilatation and regulating RAS through ACE inhibition, with some additional beneficial effects mediated by IQW alone (i.e. reducing vascular inflammation and oxidative stress). While our work validates the anti-hypertensive properties of these peptides in a relevant model system there are a few limitations associated with this study. For example, our results were obtained using chemically synthesized peptides of high purity. Whether similar effects can be observed from crude preparations containing these peptides (as well as various impurities) remains to be determined. Use of pure peptides, if justified, would have to be further evaluated for commercial viability. In addition, we used only adult male rodents with fully developed hypertension. Future studies with a longer treatment period in younger animals and also in animals of both sexes may be performed to determine the preventive effects, if any, of these peptides. Thus, these findings might justify the use of egg as a source for the development of novel functional food and/or nutraceuticals for the prevention and management of hypertension and associated cardiovascular disorders.

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Fig. 5 – IQW and LKP treatment do not restore eNOS expression in SHR vasculature. Protein levels of eNOS, normalized to β actin in mesenteric artery (A) and aortic (B) lysates from untreated, IQW and LKP treated animals. Data represented as mean ± SEM from n = 4 animals per treatment group.

Fig. 6 – IQW and LKP treatment attenuate plasma Ang II levels. Plasma Ang II (pg/mL) levels from untreated, IQW and LKP treated SHRs are shown. Data represented as mean ± SEM from n = 4 animals per treatment group. * indicate P < 0.05, as compared to the untreated group.
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